CYANIDE METABOLISM IN BACTERIA AND PLANTS

Thesis submitted by

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ABSTRACT

The metabolism of organic cyanides and amides was investigated in a species of *Rhodococcus* newly isolated from garden soil for its capacity to use acetonitrile as sole C and N source. Acetonitrile-grown cells hydrolysed a number of nitriles and amides to ammonia; although hydrogen cyanide (HCN) may have also been hydrolysed thus, the results were close to experimental uncertainty. The bacterium was shown to utilise a variety of amides as the sole source of C and/or N for growth although it could not grow with every corresponding nitrile.

Succinate/ammonium sulphate grown cells did not hydrolyse acetonitrile or acetamide indicating that the enzymes involved in nitrile degradation are subject to induction. Acetamide and acetate appeared to be gratuitous inducers of the acetonitrilase; acetate also induced the acetamidase.

Optimum conditions were sought for assaying the acetonitrilase and acetamidase activities in whole bacteria. These enzyme activities were also detected in cell-free extracts although the cells proved difficult to disrupt. The nitrilase was found to be particularly thermolabile whether assayed using whole bacteria or extracts. Freezing this enzyme in the soluble form resulted in total loss of activity. The amidase was more thermostable and cell-free extracts retained the ability to hydrolyse acetamide after being frozen although the activity was greatly reduced.

The metabolism of HCN was investigated further in a variety of higher plants including both cyanogenic and non-cyanogenic species. The enzymes investigated were β-cyanoalanine synthase, rhodanese and formamide hydro-lyase. β-Cyanoalanine synthase was found to be present in

every higher plant tested whereas rhodanese occurred far less commonly. Formamide hydro-lyase activity was not detected in any of the higher plants tested. Furthermore, a general trend was apparent between the HCN-potential of each plant and cyanide metabolising activity, in that the higher the HCN-potential, in general, the higher the cyanide metabolising activity.

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LIST OF ABBREVIATIONS

Abbreviations, except as listed below, are in accordance with the Biochemical Journal 'Policy of the Journal and Instructions to Authors' (Biochemical Journal (1978), 169, 1 - 27).

$A_{(x)}^{nm}$	absorbance of a solution at x nm in a 1cm light path
BSA	bovine serum albumin
BSM	basal salt medium
Ch	chapter
cpds.	compounds
C-source	carbon source
DCPIP	dichlorophenol-indophenol
D.D.W.	deionized distilled water
D/L	dextrorotatory and levorotatory isomers
D.W.	distilled water
Eq.	equation
FHL	formamide hydro-lyase
g.f.w.	gram fresh weight
hrs.	hours
IAA	indoleac etic acid
IAN	indoleacetonitrile
lb/in^2	pounds per square inch

m.m.	minimal medium
m.w.	molecular weight
N.A.	nutrient agar
N.C.I.B.	National Collection of Industrial Bacteria
N.D.	not detected
N.T.	not tested
O.D.	optical density
P.F.	protein fraction
PVP	polyvinylpyrrolidone
R.B.	reagent blank
r.p.m.	revolution per minute
S.A.	specific activity
T.C.A.	trichloroacetic acid
UDP-glucose	uridine diphosphate glucose

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CHAPTER I

INTRODUCTION

Cyanides are defined as organic or inorganic compounds which contain the -C≡N grouping (Towill *et al.*, 1978). In this thesis organic cyanides will be referred to, in most cases, as the nitriles of the corresponding carboxylic acids.

1.1 INDUSTRIAL USES OF CYANIDES

Both hydrogen cyanide (HCN) and its salts form complexes with metals, especially those of the transition series and it is this property which is utilised for industrial purposes. The main uses for HCN and its salts are in the electroplating and steel industries, in ore extraction, paint manufacturing, metal-cleaning operations, chemical syntheses and to a lesser extent in photographic processing (Towill *et al.*, (1978).)

For 1976, the estimated U.S. production of HCN was 312,500 tons. Of this, 52% was used in acrylonitrile production, 18% in methyl-methacrylate production, 14% in adiponitrile production, 7% as sodium cyanide and the remaining 9% for a variety of purposes such as insecticides and rodenticides (Towill *et al.*, 1978).

A number of economically important organic compounds are synthesised industrially from nitriles. Some industrially important reactions of nitriles have been listed by Jallageas *et al.*, (1980). One such reaction is hydrogenation to amines. Thus, adiponitrile is pressure hydrogenated at 150^{o}C to hexamethylene-diamine used in the synthesis of polymers. Similarly, nitriles of fatty acids are converted to the corresponding amines which are used as surfactants, emulsifiers, detergents and flotation agents.

Another important reaction is the hydrolysis of nitriles to the corresponding amides or acids. Examples include acid hydrolysis of phenyl-

acetonitrile to phenylacetic acid for perfumes; acrylonitrile to acrylamide for polyacrylamide fibres; lactonitrile to DL-lactic acid for therapeutics, dyes and tanning and of mandelonitrile to DL-mandelic acid for antispetics. Acetone cyanohydrin is also hydrolysed with acid to α-hydroxyisobutyric acid which is further converted to methacrylic acid used in the manufacture of plastics. α-Aminonitriles are converted to α-amino-acids by both acid and alkaline hydrolysis and the products used for animal feed, therapeutics, cosmetics etc. Alkaline hydrolysis is also used to convert malononitrile to malonic acid used in the production of medicaments (Jallageas *et al.*, 1980).

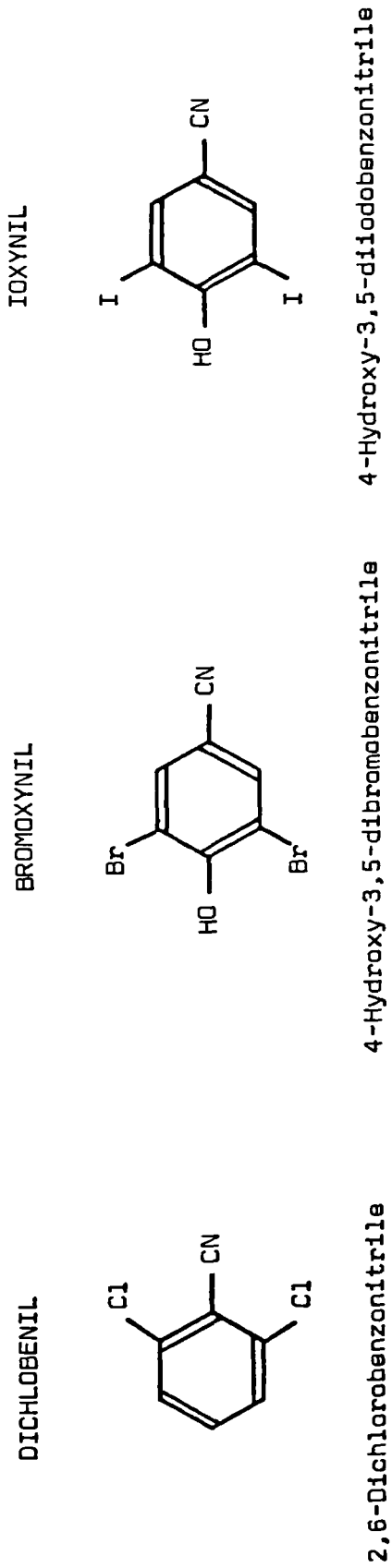
Certain halogenated aromatic nitriles are used as herbicides. The structures of the three major compounds, dichlobenil, bromoxynil and ioxynil are shown in Fig. 1.1. Dichlobenil is used as a pre-emergence herbicide. It is usually applied to the soil to inhibit germination, often to eliminate annual weeds in orchards and vineyards (Ashton & Crafts, 1973). It is active against both monocotyledons and dicotyledons but does not affect trees and established crops with deep root systems because it produces little contact damage and does not migrate appreciably into the soil. Ioxynil and bromoxynil are used as post-emergence contact herbicides to control broadleaf plants that are frequent weeds in cereal and grass crops. Both ioxynil and bromoxynil are applied as sprays when weeds are in their early stages of growth (Ashton & Crafts, 1973).

1.2 ENVIRONMENTAL OCCURRENCE OF CYANIDES

1.2.1. Occurrence Due to Man

The largest amount of inorganic cyanide containing waste is generated by the electroplating industry which contains 0.5-20% cyanide (Towill *et al.*, 1978). However, only a relatively small quantity is believed to escape

FIGURE 1.1. Structures of the Major Nitrile Herbicides.



into the environment if proper waste management is employed. Prior to disposal, wastewaters are decontaminated by one of 3 methods:-

(i) alkaline chlorination

(ii) electrolytic decomposition

(iii) ozone oxidation.

Thus the final concentration of inorganic cyanide in liquid wastes from the electroplating industry in the U.S. ranges from 9-115 ppm (Towill *et al.*, 1978). The steel and mining operations also discharge cyanide to the environment. A mineral processing plant in West Java discharges approximately 98 tons of sodium cyanide each year into the river Tjimadur (Towill *et al.*, 1978).

Ferricyanides and ferrocyanides, used for the dying of fabrics and in the heat treatment of iron and steel also occur in waste streams from industry. Although both compounds have relatively low toxicities, they can breakdown to release HCN when irradiated by the U.V. component of sunlight (Towill *et al.*, 1978).

Atmospheric emissions of HCN from petrochemical industries and plating plants do occur occasionally but more often HCN occurs in the air as a result of the fumigation of ships, warehouses and agricultural buildings where the cyanide is used as an insecticide or rodenticide. Usually the concentration of HCN used for fumigation is less than 1% (Towill *et al.*, 1978). Another source of atmospheric cyanide is from car exhaust fumes which contain approximately 1 ppm HCN together with traces of acetonitrile (Schuchmann & Laidler, 1972). Here the cyanides are formed by reaction of NO with organic free radicals present in the combustion mixture.

The combustion of nitrogen containing synthetic polymers releases HCN and nitriles (Anderson *et al.*, 1979); e.g. polyurethane liberates HCN upon pyrolysis. Dangerous concentrations of cyanides may therefore be produced in burning buildings where these polymers are widely used for

construction and furnishing. This probably explains why the proportion of fire casualties now attributed to the effects of smoke and gas inhalation has risen 3-fold over the last two decades (Anderson *et al.*, 1979). The natural cyanide level in human blood is on average 2.9μM HCN whereas in fire fatalities the concentration can be as high as 238μM HCN. In addition, propionitrile, the most toxic of the alkyl cyanides, has been identified in blood samples taken from fire victims together with two unidentified mono-unsaturated C_4-nitriles; none of which are present in normal blood (Anderson *et al.*, 1979).

Most of the artificial nitriles in the environment, however, represent losses associated with their industrial production and use; in the U.S. the annual loss of acrylonitrile is estimated to be about 7,000 tons (Towill *et al.*, 1978).

The nitrile herbicide dichlobenil which is applied to the soil is not readily leached and acts only in the top soil. Furthermore, it is volatile and quickly lost from soils (Towill *et al.*, 1978). Dichlobenil, bromoxynil and ioxynil act as electron-flow inhibitors or uncouplers in respiration and/or photosynthesis although free HCN is not formed by the breakdown of any of these nitrile herbicides (Ashton & Crafts, 1973).

1.2.2 Natural Occurrence of Cyanides

The production of HCN by bacteria has been observed for certain strains of *Pseudomonas aeruginosa*, *P. fluorescens* (Castric, 1975), *P. chloroaphis*, *P. aureafaciens*, *Chromobacterium violaceum* (Michaels & Corpe, 1965) and for an unidentified *Pseudomonas* sp. (Wissing, 1968). Maximal production of HCN in bacterial cultures occurs once the growth rate has begun to decline (Michaels & Corpe, 1965; Wissing, 1968; Castric, 1975) and is dependent on the inclusion of glycine in the medium. By

incubating cells of *C. violaceum* with 1-^{14}C and 2-^{14}C-glycine, Michaels *et al.*, (1965) demonstrated that the HCN was formed from the methylene (2-C) group of glycine whereas the carboxyl-group gave rise to CO_2.

Cyanogenesis is widespread amongst the fungi. Bach (1956) listed 31 species of fungi which form HCN; they included one species from the order *Mucorales* and several basidiomycetes from the orders *Agaricales* and *Polyporales*. Earlier, Locquin (1944) had claimed 300 species of basidiomycetes and several ascomycetes to be cyanogenic.

The HCN may be formed both by fruiting bodies, e.g. in *Marasmius oreades* (Lebeau & Hawn, 1963) and by mycelia during the autolytic phase of growth in culture, e.g. *M.oreades* (Lebeau & Hawn, 1963) and the snow mold basidiomycete (Ward & Lebeau, 1962). Mycelia of *M.oreades* are also known to produce HCN under natural conditions when growing as fairy rings in lawns (Lebeau & Hawn, 1963).

As in the case of bacteria, glycine stimulated HCN production by an isolate of the snow mold fungus: again radiolabelling studies showed that the cyanide-carbon was derived from the methylene group of glycine (Ward & Thorn, 1966). Cultures of this snow mold isolate accumulate unstable compounds which yield HCN upon autolysis (Ward, 1964). The major cyanogen was identified as glyoxylic acid cyanohydrin although lower concentrations of pyruvic acid cyanohydrin were also formed (Tapper & MacDonald, 1974).

Many higher plants are also known to be cyanogenic. More than 2050 species from 110 families including ferns, gymnosperms and mono- and dicotyledenous angiosperms can liberate HCN (Seigler, 1981). Hydrogen cyanide does not exist in the free state in the plant to any extent; instead it is produced by enzymes acting on one or more compounds that serve as precursors. These compounds are usually β-glycosides of α-hydroxynitriles and are known as cyanogenic glycosides.

These glycosides are quite common in certain families, e.g. 150 *Rosaceae* sp., 100 *Leguminosae* sp., 100 *Gramineae* sp., 50 *Araceae* sp. and 50 *Compositae* sp. are known to be cyanogenic (Towill *et al.*, 1978). The interest in these compounds, however, lies in the fact that several of the plants of mans' economy are cyanogenic.

Plant tissues commonly consumed by man or other animals that are produced by cyanogenic species include cassava tubers (*Manihot esculenta* Crantz), *Sorghum* seed and foliage, white clover foliage (*Trifolium repens*), bamboo shoots (*Banbusa vulgaris*) linseed cake (flax - *Linum usitatissimum*) and nuts of the almond (*Prunus amygdalus*) and macadamia (*Macadamia ternifolia*) trees (Conn, 1979). Legume seeds reported to be cyanogenic include the black-eyed pea (*Vigna sinensis*), common pea (*Pisum sativum*), kidney bean (*Phaseolus vulgaris*), chick pea (*Cicer arietinum*) (Montgomery, 1965) and lima bean (*Phaseolus lunatus*) (Conn, 1979). Seedlings of commercial varieties of wheat, rice and barley have also been found to be cyanogenic (Conn, 1979).

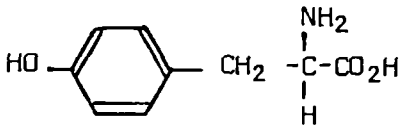
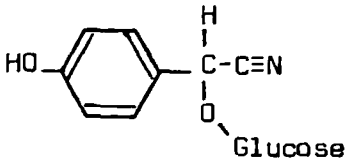
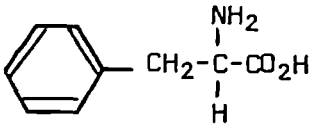
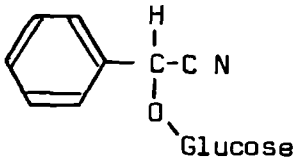
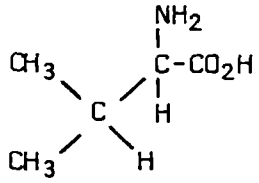
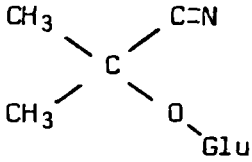
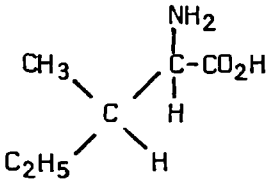
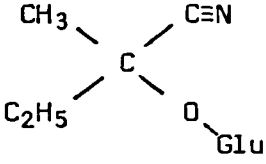
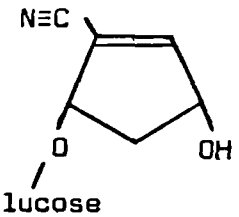
It is often the case, however, that the concentration of a cyanogenic glycoside in a plant varies in different tissues and also with age. Examples of this are the stone fruits of the edible *Rosaceae*. Although the fleshy fruit of the cultivated peach, apricot, cherry, apple and pear are commonly eaten, the seeds of these species are known to be cyanogenic (Conn, 1979).

Twenty-three different cyanogenic glycosides have been isolated from higher plants (Conn, 1979). The aglycone portion of the majority of these compounds is formed (or presumed to be formed) from a structurally related amino acid. Table 1.1 shows the structures of 4 cyanogenic glycosides related to protein amino acids and an example of one glycoside that is not.

The general pathway for the biosynthesis of cyanogenic glycosides is shown in Fig. 1.2. From this scheme it can be deduced that the nitrile intermediate formed from the precursor amino acid would be phenylacetonitrile

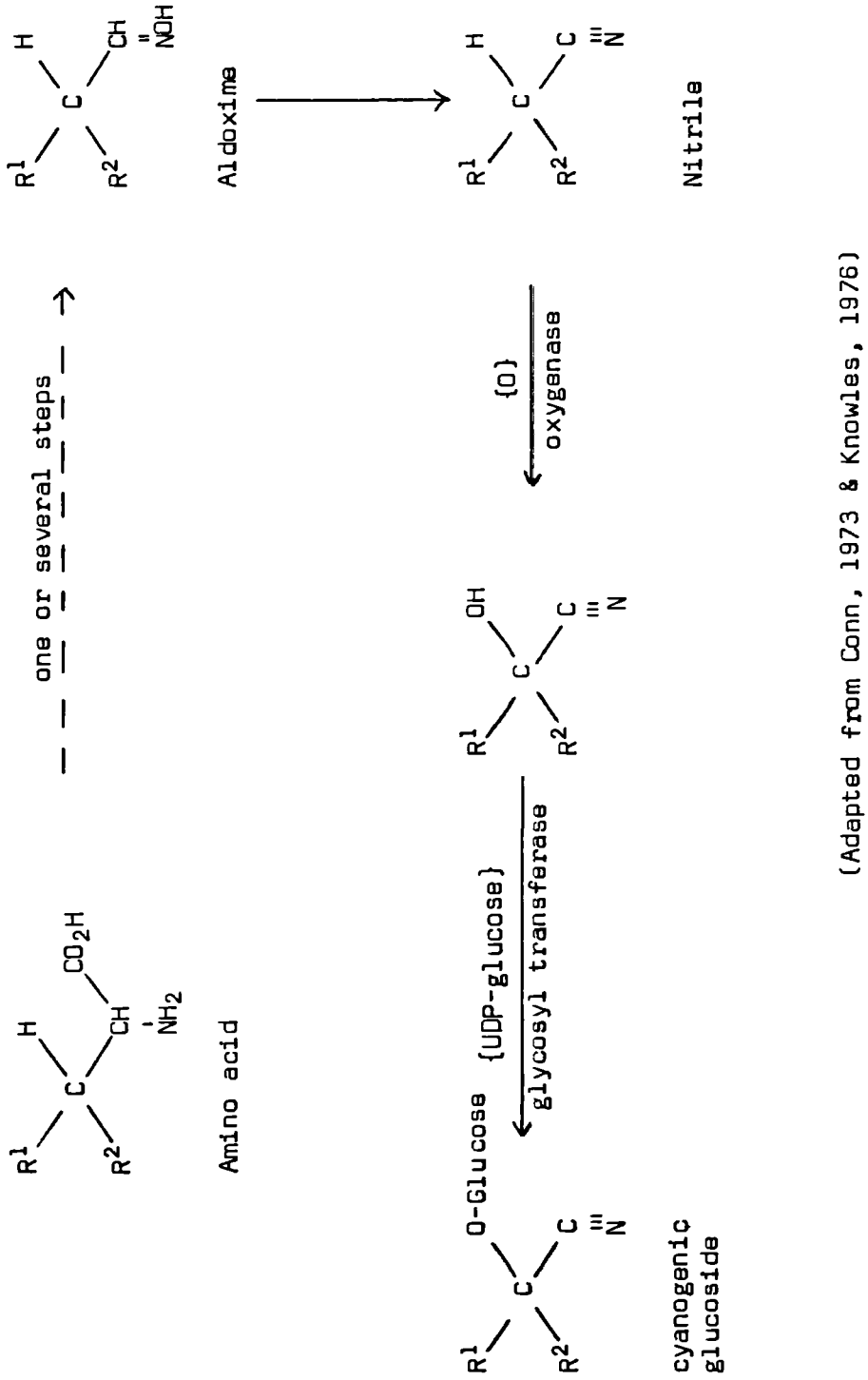
TABLE 1.1

Precursor-Product Relationships between Amino Acids and Cyanogenic Glycosides

Amino acid precursor	Cyanogenic glycoside	Examples of plants containing glycoside
		*Sorghum* sp.
(L) tyrosine (TYR)	dhurrin	
		cherry laurel, peach, almond leaves
(L) phenylalanine (PHE)	prunasin	
		linen flax cassava lima beans white clover
(L) valine (VAL)	linamarin	
		linen flax, cassava lima beans white clover
(L) isoleucine (ILE)	lotaustralin	
(L) 2-cyclopentene-1-glycine (never shown to occur in nature)	 tetraphyllin B	Members of the *Passifloraceae* e.g. *Adenia volkensii*

(Adapted from Conn, 1973 & 1979; Siegler, 1981)

Figure 1.2 The Biosynthesis of Cyanogenic Glucosides



(Adapted from Conn, 1973 & Knowles, 1976)

in the case of PHE, p-hydroxyphenyl-acetonitrile in the case of TYR, isobutyronitrile in the case of VAL and 2-methylbutyronitrile in the case of ILE.

The degradation of cyanogenic glycosides occurs in two steps (Fig. 1.3). Such reactions occur at a significant rate only after the plant has been crushed such that the cellular structure of the tissue has been destroyed, (Conn, 1973). This suggests that the degradative enzymes are spatially separated from the glycoside in the intact plant. Indeed this has recently been proved in a series of elegant experiments by Kojima *et al.*, (1979). By preparing protoplasts from the leaves of *Sorghum bicolor* it was found that the cyanogenic glycoside, dhurrin, was located entirely in the epidermal layers whereas the two enzymes responsible for its degradation resided in the mesophyll tissue.

Cyanogenesis is not confined to the plant kingdom but is also known in arthropods of the classes *Chilopoda* (centipedes), *Diplopoda* (millipedes) and *Insecta* such as beetles. Here precursors of HCN include the cyanogenic glycosides linamarin and lotaustralin detected in one species of moth (*Zygaena filipendulae*) and a few species of butterflies. Other possible precursors such as p-isopropylmandelonitrile glycoside, mandelonitrile, mandelonitrile benzoate and benzoylcyanide have also been isolated (Duffey, 1981).

Although cyanogenic glycosides are the precursors of HCN in most cases, a limited number of plants have been shown to contain cyanolipids. These compounds are only known to occur in the family *Sapindaceae*. Cyanolipids differ from cyanogenic glycosides in that the sugar moiety is replaced by a fatty acid. The 4 known cyanolipids are all derived from leucine although only 2 of these liberate HCN upon hydrolysis (Siegler, 1981) (Fig. 1.4).

Figure 1.3. The Degradation of the Cyanogenic Glycoside Linamarin

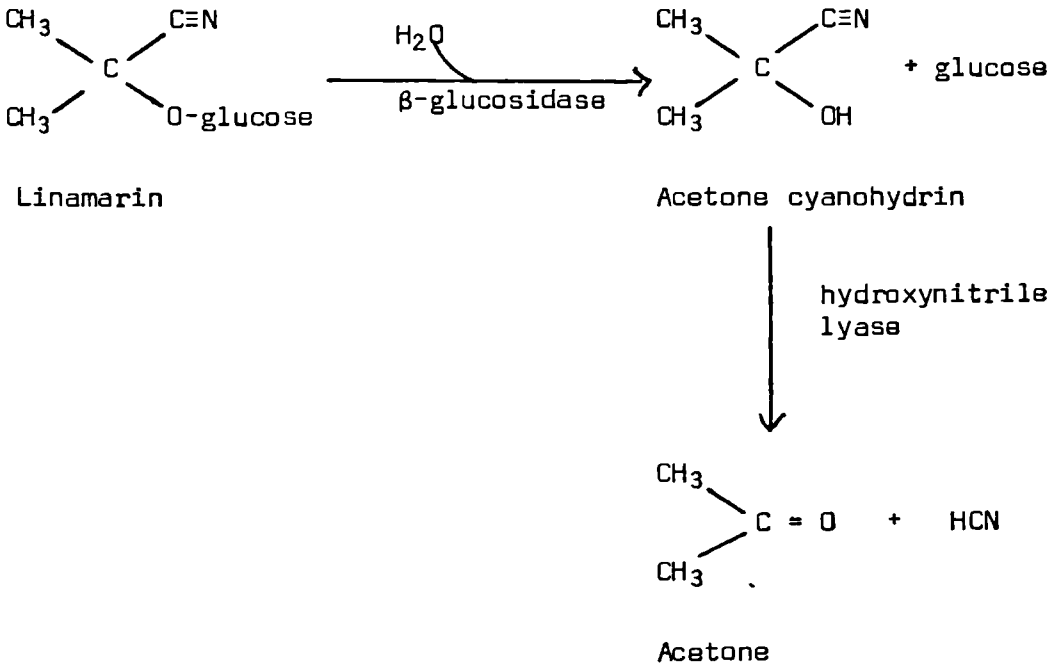
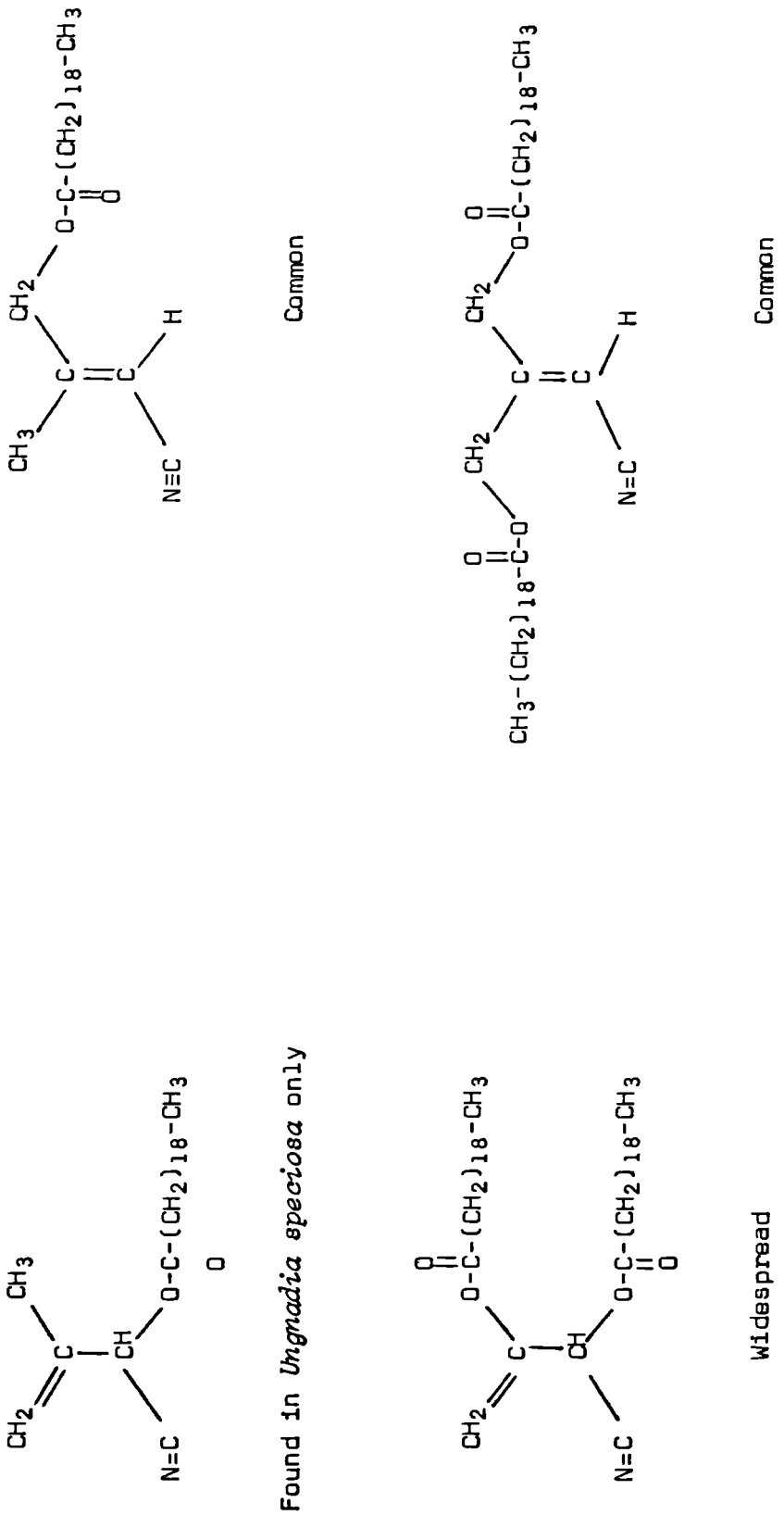
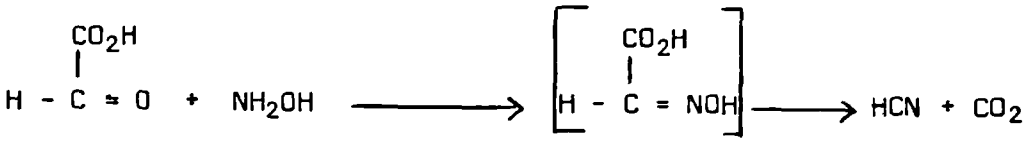


Figure 1.4. The Structures of Cyanolipids Present in the *Sapindaceae*.



Cells of *Chlorella vulgaris* have been shown to contain HCN (Gewitz *et al.*, 1974). The proposed pathway for cyanogenesis in this algae is as follows:

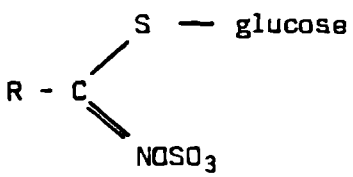


(Solomonson & Spehar, 1977).

In *C. vulgaris*, HCN is thought to act as a regulator of nitrate reductase together with NADH or NADPH. The inactive nitrate reductase present in ammonia treated *Chlorella* cells releases a stoichiometric amount of HCN upon activation with nitrate (Lorimer *et al.*, 1974). Thus the role of HCN and NAD(P)H in the reversible inactivation of nitrate reductase is thought to involve the formation of a firmly bound complex of reduced enzyme and HCN : E_R-HCN, (Lorimer *et al.*, 1974).

Gewitz *et al.*, (1974) applied their bioassay and chemical analyses, developed with *C. vulgaris*, to spinach leaves and to the cyanobacterium *Plectonema boryanum*, neither of which are known to be cyanogenic. Due to the sensitivity of these analyses they were able to demonstrate the presence of HCN in both these plants. These results led Hegnauer in 1976 to write '.....consequently prussic acid may be present in all plants,' (cited by Jones, 1979).

Glucosinolates (mustard oil glucosides) are a group of secondary plant products found in members of the family *Cruciferae*. These compounds, like the cyanogenic glycosides and cyanolipids, are thought to be synthesised from aminoacids and are broken down when the plant is crushed. Glucosinolates have the general structure,

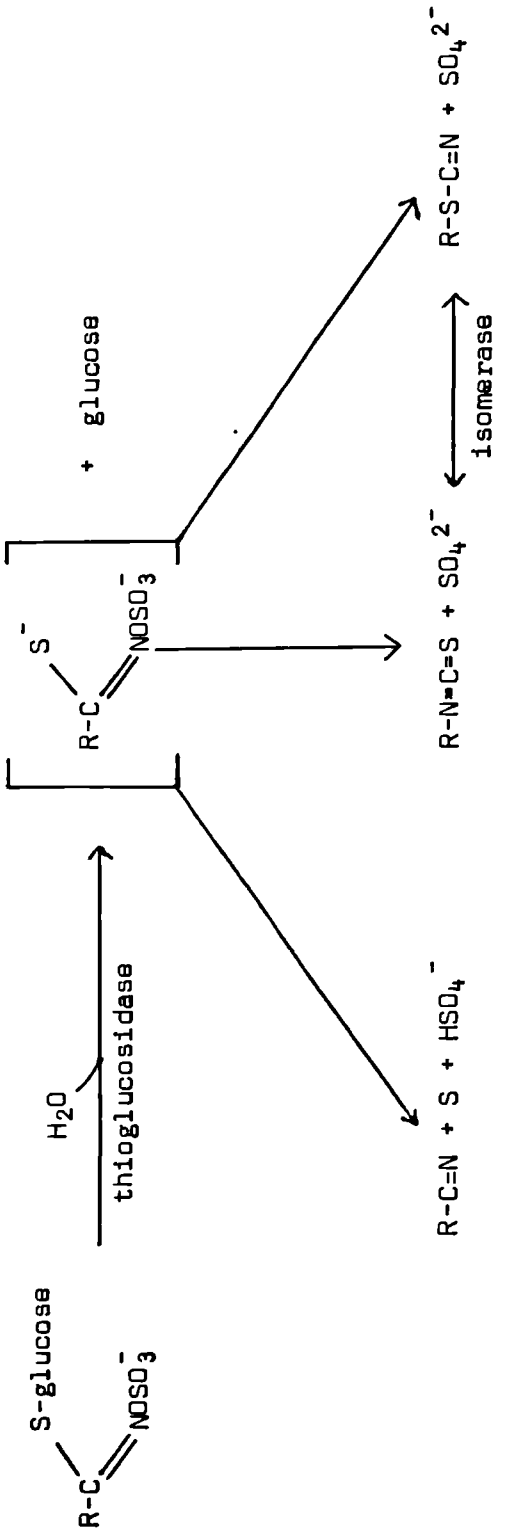


and are degraded by hydrolytic enzymes known as thioglucosidases (myrosinases) to yield nitriles, isothiocyanates or thiocyanates (Fig. 1.5). The nature of the products formed is often pH dependent low pH favouring nitrile production (Benn, 1977). Thus at pH 3-4, glucobrassicin (where R = 3-indolylmethyl in the above structure) is converted to indoleacetonitrile (Towill *et al.*, 1978). However, when the seed powder of *Lepidium sativum* (garden cress) is moistened, phenylacetonitrile, benzylisothiocyanate and benzylthiocyanate are all formed from the parent glucosinolate glucotropaeolin (Virtanen, 1965). Nitrile formation can be promoted by metal cations such as Fe^{2+}; this observation may account for some of the high nitrile yields obtained from plant and seed preparations under pH conditions expected to favour isothiocyanate formation, i.e. neutral/alkaline (Benn, 1977). At present, 50 glucosinolate compounds are known (Towill *et al.*, 1978).

3-Indoleacetonitrile (IAN) was isolated from cabbage and brussel sprouts by Jones *et al.*, (1952). Neutral extracts of other members of the *Cruciferae* (eg radish and cauliflower) were also shown to have growth promoting activity suggesting that the nitrile was more widely distributed. However, since glucobrassicin is known to occur in these plants IAN may be an artefact (Ferris, 1970).

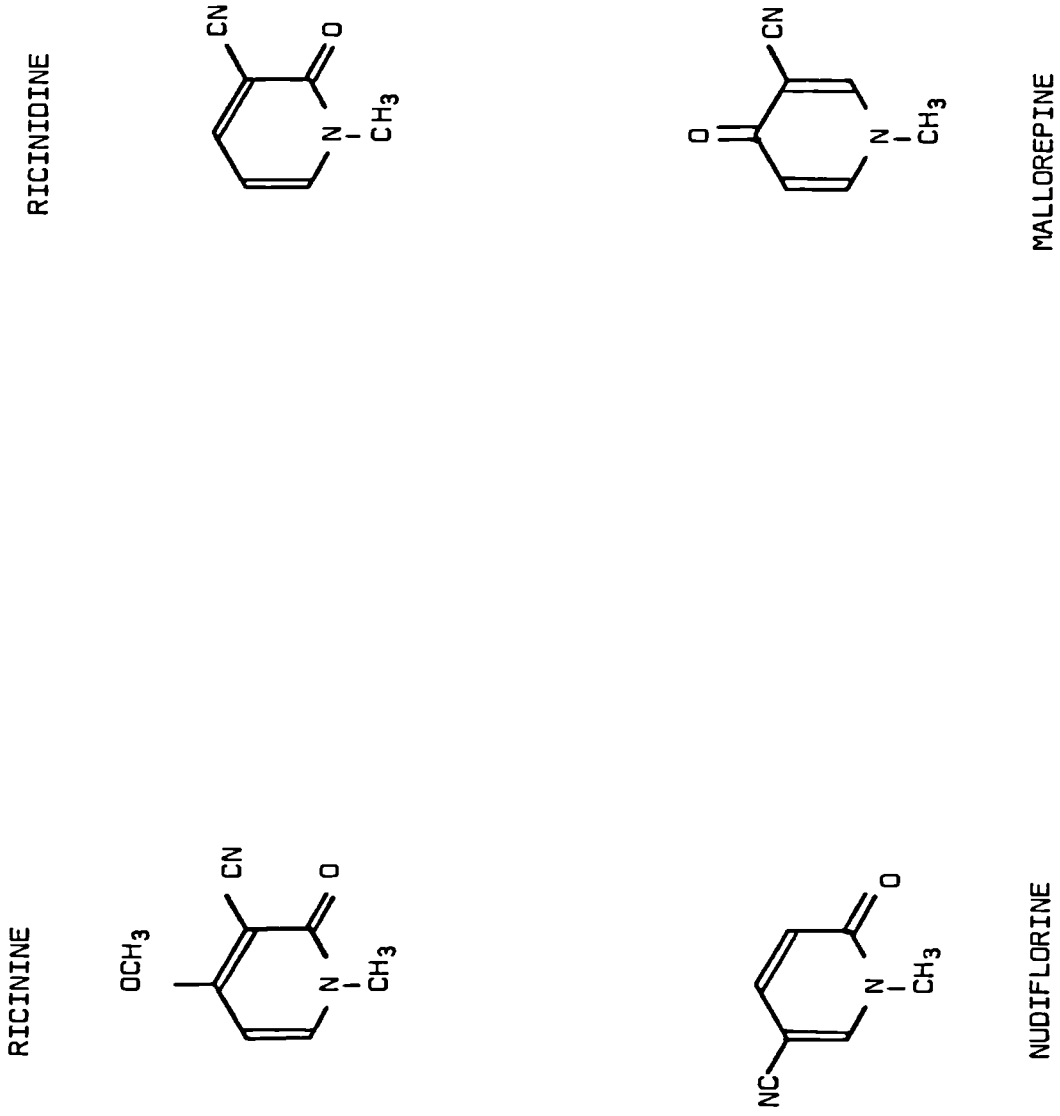
Three cyanopyridine alkaloids have been isolated from 2 plants belonging to the family *Euphorbiaceae*. Ricinine has been isolated from *Ricinus communis* (castor bean) and ricinidine and nudiflorine from *Trewia nudiflora* (Johnson & Waller, 1974; Ganguly, 1970). The structures of these alkaloids are given in Fig. 1.6. A fourth cyanopyridine derivative, mallorepine, has been isolated from another member of the *Euphorbiaceae*, namely *Mallotus repandus*, and is thought to be an intermediate in the proposed biosynthetic pathway from nicotinamide to ricinine (Hikino *et al.*, 1978).

Figure 1.5 General Scheme for the Degradation of Glucosinolates.



(Adapted from Bann, 1977 and Towill *et al.*, 1978)

FIGURE 1.6. Structures of the Cyanopyridine Alkaloids.



1.3 CYANIDE METABOLISM

Several enzymes are known to be involved in the metabolism of HCN. One of them, β-cyanoalanine synthase (EC 4.4.1.9) catalyses the following reaction:

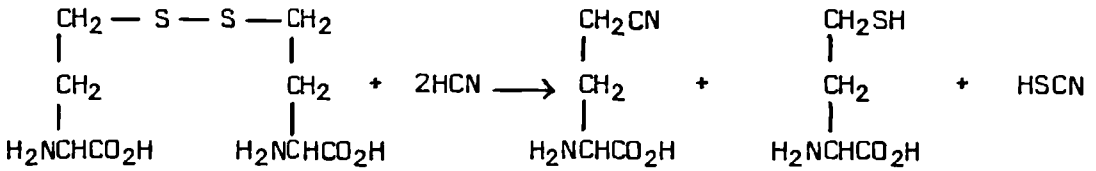
$$HSCH_2CHNH_2CO_2H + HCN \longrightarrow NCCH_2CHNH_2CO_2H + H_2S$$

cysteine β-cyanoalanine

The activity has been demonstrated in a variety of higher plants including *Sorghum* sp., common vetch, barley, safflower, flax, mung beans (Floss *et al.*, 1965) and blue lupine (Hendrickson & Conn, 1969). This enzyme is also found in bacteria, e.g. *Escherichia coli* (Dunnill & Fowden, 1965), *Bacillus megaterium* (Castric & Strobel, 1969) and *C. violaceum* (Brysk *et al.*, 1969).

Extracts of plants (Floss *et al.*, 1965) and of bacteria (Dunnill & Fowden, 1965) can also use serine as an alternative to cysteine in the above reaction. However, it was shown with extracts of *Lotus tenuis* that the rate of β-cyanoalanine formation, in the presence of HCN, was 50 times greater with cysteine than with serine (Floss *et al.*, 1965). In plants the enzyme is located in the mitochondria (Floss *et al.*, 1965; Hendrickson & Conn, 1969), a finding which has led to speculations that its function in cyanogenic plants may be to protect the cytochrome system by detoxifying HCN. However, since its occurrence is not restricted to such plants its physiological significance remains obscure.

In addition to β-cyanoalanine formation the cyanogenic bacterium *C. violaceum* can also metabolise cyanide to γ-cyano-α-aminobutyric acid (Brysk & Ressler, 1970). The relevant enzyme was purified and found to catalyse the following reaction:

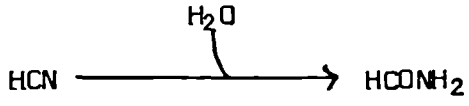


homocystine | γ-cyano-α-aminobutyric acid | homocysteine

(Ressler *et al.*, 1973).

Several pathways are known in fungi in which HCN reacts with an aldehyde to ultimately yield an aminoacid. The snow mold basidiomycete has been shown to metabolise HCN by two such routes involving acetaldehyde and succinic semialdehyde (Strobel, 1966; 1967), (Fig. 1.7). In an analogous way the non-cyandgenic fungus *Rhizoctonia solani* can react HCN with propionaldehyde (Mundy *et al.*, 1973), (Fig. 1.7). The first step in these reactions presumably involves the formation of a cyanohydrin which may or may not be enzymatically driven.

The fungus *Stemphylium loti* is a pathogen of the cyanogenic plant *Lotus corniculatus* which releases HCN upon infection. The fungus can tolerate a cyanide containing environment since it posesses both a cyanide-insensitive alternate respiratory system (Rissler & Millar, 1977) and an inducible enzyme capable of detoxifying HCN (Fry & Millar, 1972). This enzyme, formamide hydro-lyase (FHL) (EC 4.2.1.66), catalyses the following reaction:



FHL activity is not however restricted to fungal pathogens of cyanogenic plants. In a survey by Fry & Evans (1977), 1 of 6 fungi not pathogenic to plants, 9 out of 14 pathogens of non-cyanogenic plants and all 11 pathogens of cyanogenic plants tested could synthesise FHL.

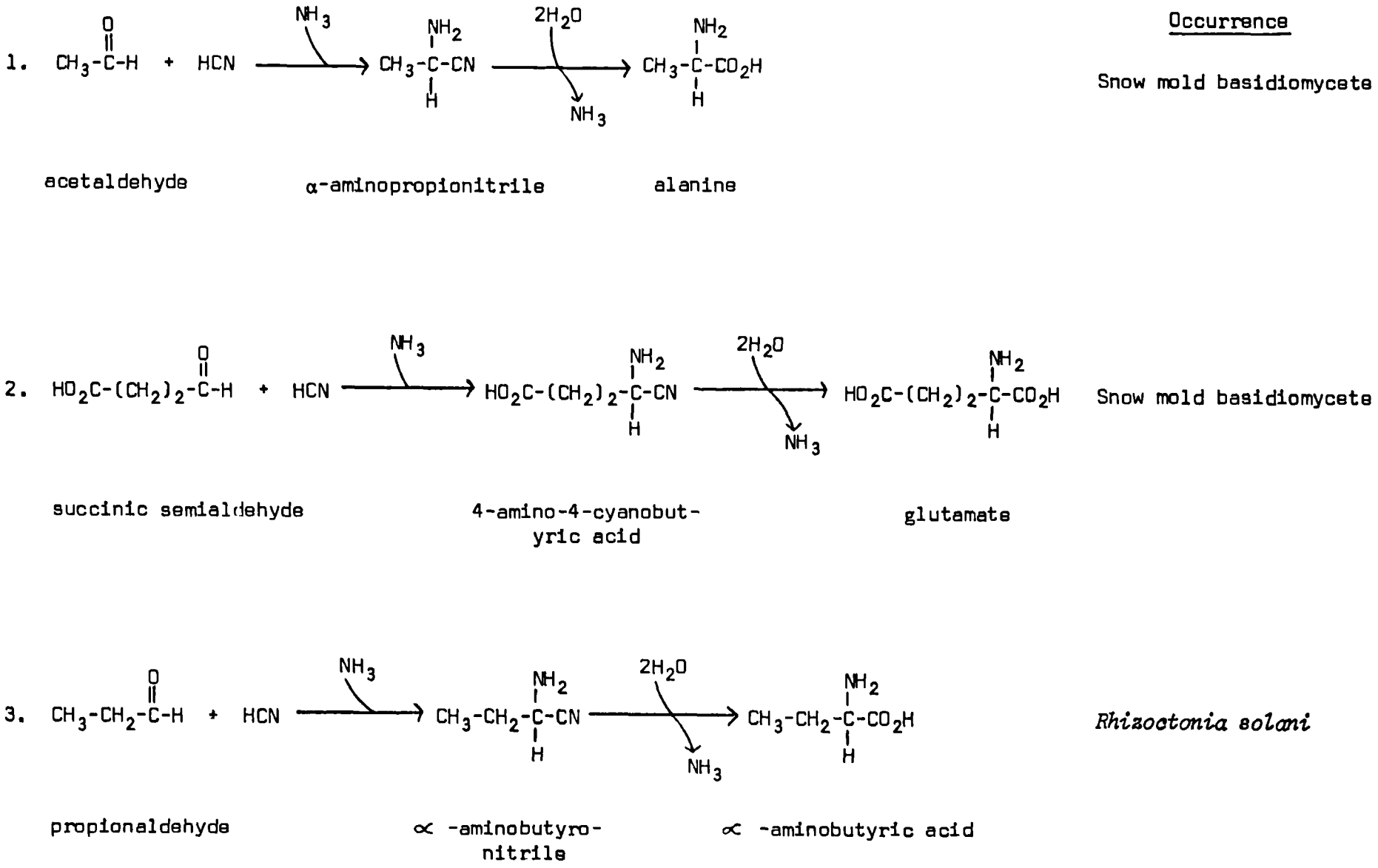


Figure 1.7 The Metabolism of HCN in Fungi by Reaction with Aldehydes

In *S.loti* and *Gloeocercospora sorghi* the conversion of HCN to formamide was stoichiometric (Fry & Millar, 1972; Fry & Munch, 1975) and further metabolism of the amide was not observed (Fry & Myers, 1981). *Fusarium solani*, however, releases ammonia from HCN which suggests a pathway involving the complete hydrolysis of HCN through to formic acid (although no product other than NH_3 was characterised) (Shimizu & Taguchi, 1969).

Rhodanese (thiosulphate : cyanide sulphur transferase EC 2.8.1.1) has been extensively studied and catalyses reactions of the kind:

$$\{RSO_xS\}^- + \text{thiophilic anion} \rightleftharpoons \{RSO_x\}^- + \text{thiolated anion.}$$

In this reaction, R can be an aryl or alkyl residue or O^- and x is an integral value in the range 0-2. Thus the sulphur donor $\{RSO_xS\}^-$ can be practically any sulphane-containing anion. The acceptor molecule can be any of a number of thiophiles including cyanide, dihydrolipoate or sulphite (Westley, 1981).

The most famous reaction catalysed by rhodanese is:-

$$S_2O_3^- + HCN \rightleftharpoons SO_3^- + HSCN$$

The enzyme has been reported to occur in fungi, various bacteria, all phyla of the animal kingdom (Westley, 1981) and in a few higher plants including cassava (Chew & Boey, 1972) and cabbage (Tomati *et al.*, 1972). The highest levels of rhodanese in mammals occurs in the liver where the enzyme has been shown to be present exclusively in the mitochondria (Koj *et al.*, 1975).

The metabolism of organic nitriles usually follows one of two routes. One type of reaction involves the cleavage of the C-C bond to yield inorganic cyanide; one such reaction, the splitting of the aglycone portion from a cyanogenic glycoside to the aldehyde/ketone plus HCN, has already been discussed. The other type of reaction involves the hydrolysis of the nitrile

group.

Enzymes catalysing the hydrolysis of nitriles are of two types. In some cases the nitrile is hydrolysed directly to the acid, using 2 molecules of water, whereas in other instances the nitrile is hydrolysed first to the amide which may then be further hydrolysed by an amidase.

The hydrolysis of IAN by plants is an example of the first type. In 1964 Thimann & Mahadevan demonstrated the presence of indoleacetonitrilase in members of the *Gramineae*, *Cruciferae* and *Musaceae* (banana family). The enzyme was not widespread however since it was absent from members of 18 other families of higher plants tested. Indoleacetonitrilase activity was also detected in several species of *Fusarium* and in *Aspergillus niger* and *Penicillium chrysogenum* (Thimann & Mahadevan, 1964). The partially purified enzyme from barley (*Hordeum vulgare*) converted IAN quantitatively to indoleacetic acid (IAA) and ammonia. Indoleacetamide was neither an intermediate in this reaction nor could it act as a substrate.

Other reactions involving the direct hydrolysis of a nitrile to the corresponding acid (plus ammonia) include conversion of benzonitrile to benzoic acid by *Nocardia rhodochrous* (N.C.I.B. 11216) and *Fusarium solani* (Harper, 1977a, b), and of ricinine to N-methyl-3-carboxy-4-methoxy-2-pyridone by an unidentified *Pseudomonas* sp. (Hook & Robinson, 1964). In the latter case, although traces of the amide (N-methyl-3-carboxamide-4-methoxy-2-pyridone) were detected when a crude extract or the purified enzyme was incubated with ricinine, it could not serve as a substrate for the enzyme.

β-Cyanoalanine hydrolase, an enzyme catalysing the formation of asparagine (ASN) from β-cyanoalanine, has been demonstrated in extracts of *Sorghum* and blue lupine (*Lupinus augustifolia*), (Castric *et al.*, 1972). These two plants however appeared to lack asparaginase activity. Although common vetch (*Vicia sativa*) had been shown to possess β-cyanoalanine

synthase, extracts of this plant did not contain β-cyanoalanine hydrolase activity (Castric *et al.*, 1972). Instead, in this plant, β-cyanoalanine combines with glutamate to form the peptide N-(γ-glutamyl)-β-cyanoalanine catalysed by the enzyme glutamyl transferase (Fowden & Bell, 1965).

Investigations into the metabolism of acetonitrile by bacteria suggest that the pathway involves formation of acetamide and then of acetic acid plus ammonia. In 1969, Mimura *et al.*, found that ammonia and an amide, not identified specifically as acetamide, accumulated in the medium when *Corynebacterium nitrilophilus* nov. sp. C-42 was grown on acetonitrile. The first step in the pathway was confirmed by showing transfer of ^{14}C from acetonitrile to acetamide in another nitrile degrader, a *Pseudomonas* sp. (Group III; N.C.I.B. 10477) (Firmin & Gray, 1976). Thus Firmin & Gray (1976) proposed that the hydrolysis of acetonitrile to acetate and ammonia involves two enzymes acting sequentially; a nitrilase (or nitrile hydratase) which converts the nitrile to the amide and an amidase which converts the amide to the acid plus ammonia.

Another example involving the microbial conversion of a nitrile to the corresponding amide is the hydrolysis of 2-2-diphenyl-3-(1-pyrrolidino)-propionitrile (an analogue of the analgesic methadon) by *Penicillium* A-9 NRRL 3946. In this instance the amide was not converted to the corre ponding carboxylic acid (Theriault *et al.*, 1972). Another fungus, *Sterigmatocystis nigra*, can hydrolyse cyanamide to urea which is then further hydrolysed by the enzyme urease to carbon dioxide and ammonia (Lamaire & Brunel, 1951).

Torulopsis candida (GN 405) was shown to convert L-α-hydroxynitriles to DL-α-hydroxyacids, although whether one or more enzymes were involved in this reaction was not determined (Fukuda *et al.*, 1973). Earlier, Fukuda *et al.*, (1971) had observed that *Corynebacterium* HR3 cells would hydrolyse α-aminonitriles to α-aminoacids. DL-α-Aminopropionitrile and DL-α-amino-isovaleronitrile were converted to DL-alanine and DL-valine respectively.

L-Valineamide was also converted to valine so it is possible that here the hydrolysis of α-aminonitriles involves the formation of an amide intermediate.

Bacterial amidases have been studied in great detail in certain species of *Pseudomonas* (Clarke, 1970; 1972), though these organisms are not known to utilise nitriles. In particular the inducible aliphatic amidase from *P. aeruginosa* is known to catalyse the following series of reactions:

Amide hydrolysis	$RCONH_2 + H_2O \longrightarrow RCO_2H + NH_3$
Ester hydrolysis	$RCO_2R' + H_2O \longrightarrow RCO_2H + R'OH$
Amide transfer	$RCONH_2 + NH_2OH \longrightarrow RCONHOH + NH_3$
Acid transfer	$RCO_2H + NH_2OH \longrightarrow RCONHOH + H_2O$
Ester transfer	$RCO_2R' + NH_2OH \longrightarrow RCONHOH + R'OH$

(From Clarke & Richmond, 1975).

Amidase enzymes of this nature are therefore more correctly termed 'aliphatic acyltransferases' as suggested by Kelly & Kornberg (1964).

1.4. TOXICITY OF CYANIDES

The metal complex forming tendency of inorganic cyanide, already mentioned in section 1.1, is the main reason for its toxicity. It can form stable complexes with various metals in enzymes; the most sensitive metalloenzyme probably being cytochrome oxidase. Other haem-containing enzymes subject to cyanide inhibition include catalase and peroxidase as well as non-haem metalloenzymes, e.g. tyrosinase (Towill *et al.*, 1978). Inorganic cyanide may also inhibit enzymes by combination with carbonyl groups of aldehydes or ketones to form cyanohydrins or by its reaction with disulphide bonds.

The tolerance of different organisms to inorganic cyanide varies widely, e.g. some bacteria are killed by exposure to less than 1 ppm cyanide whereas a strain of *Bacillus pumilus* could survive in a saturated solution of KCN (*Towill et al.*, 1978; Skowronski & Strobel, 1969).

The toxicity of many organic nitriles often appears at first to be due to the particular structural characteristic of the whole molecule concerned rather than by an initial decomposition to release HCN. For example, β-cyanoalanine and its N-(γ-glutamyl) derivative found in *V. sativa* act as neurotoxins in mammals (Ferris, 1970) whereas β-aminopropionitrile is teratogenic (Towill *et al.*, 1978). Toxicity can be a result of the breakdown of the nitrile to release HCN however as in the case of the cyanogenic glycosides.

1.5 AIMS OF PROJECT

One of the aims of this project was to investigate, in more detail than any previous worker, the metabolism of nitriles by bacteria. The primary objectives were to acquire fundamental information about the following:

(i) the range of nitriles and amides capable of supporting bacterial growth,

(ii) the substrate specificity of the enzymes responsible for nitrile and amide degradation,

(iii) the biosynthetic control of the nitrilase and amidase enzymes.

These objectives necessitated the development of a quick technique for assaying nitrilase and amidase activities. Furthermore, by optimising conditions for these assays knowledge about the properties of the nitrilase and amidase enzymes in nitrile-degrading bacteria would be obtained for the first time.

The second aim of this project was to investigate the occurrence and distribution of HCN metabolising enzymes in higher plants. Two of the enzymes chosen for this survey were rhodanese and FHL since neither had been extensively investigated in higher plants before. The activity of these two enzymes,together with β-cyanoalanine synthase was measured in a variety of higher plants including both cyanogenic and non-cyanogenic species.

Since 1976 considerable interest has arisen in the microbial conversions of nitriles, in particular that of acetonitrile. Thus a number of relevant publications have appeared during the course of this project. These have been discussed in the appropriate chapters and compared with the results obtained here.

CHAPTER 2

MATERIALS AND METHODS

2.1. GENERAL

Chemicals

Chemicals were of 'reagent' grade and usually supplied by British Drug Houses Ltd., Poole, Dorset, except for the experiments detailed in Chapter 3 for which 'analar' reagents were used and supplied by Mallinckrodt, St. Louis, MO., U.S.A. All reagents were dissolved in distilled water (D.W).

Washing up of Glassware

Glassware was washed in dilute teepol and rinsed 6 times in tap water followed by 3 times in D.W. If reagents containing heavy metals had been used in a previous experiment the glassware was soaked in chromic acid (>2 hours) prior to being washed as described above. Chromic acid was prepared by adding 940mls of concentrated sulphuric acid carefully to 35g of potassium dichromate in 60mls D.W.

2.2. PREPARATION OF MEDIA AND GROWTH OF MICROORGANISMS.

2.2.1. Sterilisation Techniques.

Sterilisation by Dry Heat.

Glassware, e.g. pipettes, was placed in an oven at 160^{o}C for a minimum of 3 hours.

Autoclaving

Aqueous media were sterilised by autoclaving at a gauge pressure of 15 lb/in^{2} (100 KPa) for 15 minutes.

Membrane Filtration.

Membrane filters (from Oxoid; pore size 0.45µm) were sterilised by

autoclaving *in situ* in plastic 'Millipore' holders wrapped in aluminium foil.

2.2.2. Preparation of Media.

The term 'minimal medium' (m.m.) is used to describe a medium containing all the nutrients needed to support the growth of the micro-organism concerned. The term 'basal salt medium' (BSM) is used to describe the inorganic salt components of the minimal medium.

(i) Minimal Medium for Growth of the *Rhodococcus* sp.

The BSM was a modification of that previously described by Firmin (1973). The medium contained: KH_2PO_4, 3.0g; K_2HPO_4, 7.0g; $MgSO_4.7H_2O$, 0.1g; 1ml of a 1% (w/v) solution of $CaCl_2.2H_2O$; 5mls of trace element solution and D.W. to 1 litre. The trace element solution contained the following compounds made up to 1 litre with D.W.: $FeSO_4.7H_2O$, 0.5g; $ZnSO_4.7H_2O$, 0.5g; $MnSO_4.5H_2O$, 0.5g; H_2SO_4(0.05M), 10mls. The corresponding m.m. was prepared by adding acetonitrile (final concentration 0.1-0.25% v/v); sodium acetate (when present) was added to a final concentration of 0.5% (w/v).

Small volumes of the m.m. were sterilised by autoclaving in sealed universal bottles to avoid possible loss of the volatile nitrile (Firmin and Gray, 1976). When cool, the medium was transferred to sterile conical flasks in a microflow cabinet. An investigation into the stability of acetonitrile was carried out by Firmin (1973) who found no detectable chemical breakdown of the nitrile in the culture medium after autoclaving, as determined by gas chromatography.

Cultures of the bacterium on a large scale (5 litres) were grown in a 15 litre capacity aspirator (Fig. 2.1.). The BSM (plus acetate when

present) was autoclaved in the aspirator in the absence of $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$. The $CaCl_2.2H_2O$ (1% w/v in D.W.), $MgSO_4.7H_2O$ (10% w/v in D.W.) and acetonitrile (10% v/v in D.W.) were sterilised separately by membrane filtration and then added to the autoclaved medium.

For the preparation of solid medium, purified agar (Difco Laboratories), 2% (w/v), was added to the m.m. before this was autoclaved in sealed universal bottles.

(ii) Minimal Medium for Growth of *Pseudomonas putida* (PPE 1)

The BSM contained: KH_2PO_4, 3.0g; K_2HPO_4, 7.0g; $MgSO_4.7H_2O$, 0.1g; 5mls of trace element solution and D.W. to 1 litre. The trace element solution contained the following compounds made up to 1 litre with D.W.: $FeSO_4(NH_4)_2SO_4.6H_2O$, 116mg; H_3BO_3, 232mg; $CoSO_4.7H_2O$, 95.6mg; $CuSO_4.5H_2O$, 8mg; $MnSO_4.5H_2O$, 8mg; $(NH_4)_6Mo_7O_{24}.4H_2O$, 22mg and $ZnSO_4.7H_2O$, 174mg. The corresponding m.m. was prepared by adding acetamide to give a final concentration of 0.5% (w/v).

The BSM was autoclaved in the absence of $MgSO_4.7H_2O$. This and acetamide (both 10% w/v BSM) were added to the autoclaved medium by sterile membrane filtration. For the preparation of solid medium, purified agar (Difco Laboratories), 1.5% (w/v), was added to the complete m.m. and autoclaved in sealed universal bottles.

(iii) V-8 Juice Medium for Growth of *Gloeocercospora sorghi**.

V-8 juice medium was prepared as previously described by Miller (1955) and Myers and Fry (1978). Thus, V-8 juice was centrifuged at 12,100g for 20 minutes and the supernatant fraction retained. To 200mls

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**G. sorghi* was a gift from Dr. W.E. Fry, Department of Plant Pathology Cornell University, Ithaca, NY 14853.

of this solution was added 250mls of 0.2M sodium phosphate buffer (pH 7.0) and D.W. to 1 litre. The medium was autoclaved in conical flasks plugged with non-adsorbent cotton-wool.

2.2.3. Growth and Maintainance of Microorganisms.

Bacterial growth in liquid medium was determined by measuring the absorption of the culture at 640nm.

(i) The *Rhodococcus* sp.

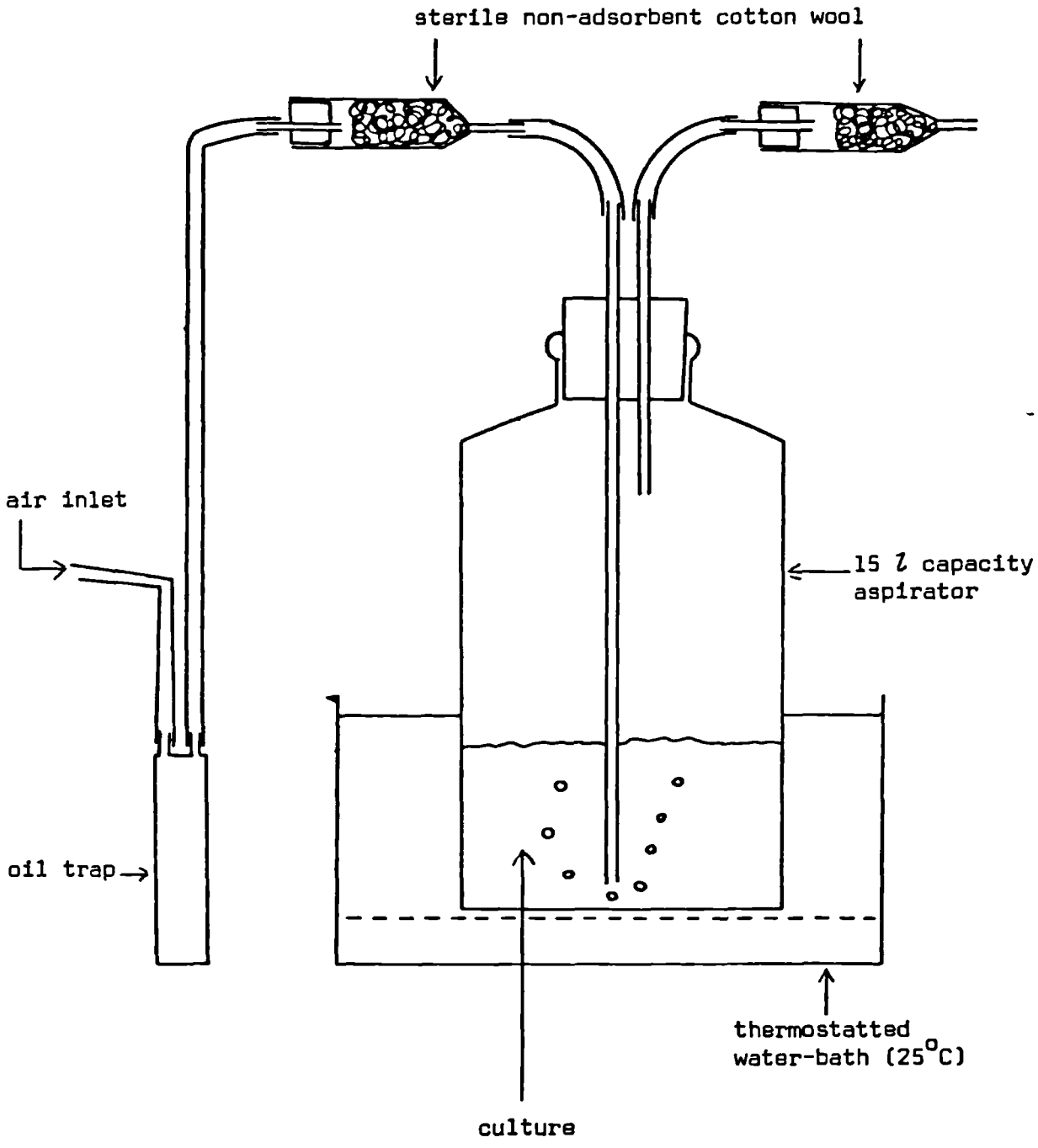
The bacterium was maintained on agar slopes and plates composed of m.m. containing acetonitrile (0.1% v/v) and sodium acetate (0.5% w/v) and subcultured at approximately monthly intervals. Cultures were initially grown for 1 week at room temperature and then stored at $4^{o}C$.

For the majority of experiments involving the *Rhodococcus* sp., starter cultures were inoculated by transferring a loop of bacteria from an agar plate into m.m. (5 x 50mls in 250ml conical flasks) containing (a) 0.25% (v/v) acetonitrile as the sole source of C and N or (b) 0.1% (v/v) acetonitrile as the sole source of N plus sodium acetate (0.5% w/v) as an additional C-source. These starter cultures were grown at $25^{o}C$ for 3 days on a linear shaker and then all transferred to 4750mls of the same medium in the aspirator (Fig. 2.1). Cultures growing in the aspirator at $25^{o}C$ were vigorously aerated with sterile filtered air supplied by a Hy-flo pump (Medcalf Bros. Potters Bar, England) and harvested during the mid/late log phase of growth.

(ii) *Pseudomonas putida*.

P. putida, strain PPE 1, was maintained on nutrient agar slopes and subcultured at monthly intervals. Cultures were grown up and stored as for the *Rhodococcus* sp.

FIGURE 2.1. Large Scale Culture Apparatus for the *Rhodococcus* sp.



For experiments involving *P. putida*, starter cultures were inoculated by transferring a loop of bacteria from a slope into 50mls m.m. (see Section 2.2.2). After 2 days growth at 25°C on a linear shaker, 5ml samples of starter culture were transferred to 95ml volumes of fresh medium in 250ml conical flasks. The cutlures were returned to the shaker and grown until reaching approximately mid-log phase.

(iii) *Gloeocercospora sorghi*.

G. sorghi was supplied on slopes of solidified V-8 juice medium. The slopes were stored at 4°C and used within 2 months of receipt.

Liquid cultures were inoculated by transferring a loop of the fungus from a slope into 25mls V-8 juice medium (in a 125ml conical flask). Flasks were shaken at 22-23°C for 9 days by which time the fungus had produced a large mycelium and was sporulating. On the 8th day, 17 hours prior to harvest, 0.25mls of KCN (0.1M in 0.05M Tris-HCl, pH 8.0) was added aseptically to half the flasks such that the final concentration in the medium was approximately 1mM.

2.3. GROWTH OF HIGHER PLANTS.

Seeds were soaked in aerated water for approximately 16 hours and then placed on damp vermiculite, except in the case of *Sorghum bicolor* (Linn.) moench where seedlings were grown on cheesecloth. Seedlings were generally grown in the dark at room temperature. For *Phaseolus lunatus* however, seed of Costa Rican wild lima beans were germinated and grown in vermiculite in the light in a growth chamber. The lima bean plants were watered every 2-3 days and a minimal salts nutrient medium (Hoaglands' solution) applied to the vermiculite once a week.

Samples of *Nandina domestica* and *Eryobotrya japonica* Lindl, were obtained from plants growing in the Davis campus. Mature leaves of *Brassica*

oleraceae var. capitata were obtained from local markets in Davis.

2.4. PREPARATION OF CELL-FREE EXTRACTS.

2.4.1 Microbial Extracts.

The preparation of all microbial extracts were carried out between 0-4^{o}C.

Bacterial extracts were usually prepared by ultrasonic disruption with a Dawe sonicator type 1130A or 7532A. The pre-cooled bacterial suspension (40-50mls) was placed in a rosette cell in an ice/water bath and sonicated for one minute intervals; the output for the type 7532A model was 90-95 watts. During each interval (1 minute) the probe was cooled in an ice/water mixture and rinsed with cold D.W. To obtain a cell-free extract, the sonicated suspension was centrifuged at 17,400g for 15 minutes at 4^{o}C and the supernatant fraction (hereafter termed the 'soluble' fraction) retained. In some experiments the pellet, presumably consisting of cell debris and unbroken cells, was resuspended in buffer at 4^{o}C (to the same volume as that sonicated) and hereafter termed the 'particulate' fraction.

Cultures of *G. sorghi* were harvested by filtration and washed with 0.05M Tris-HCl buffer (pH 8.0) and then resuspended in fresh buffer of similar composition. Extracts of *G. sorghi* were prepared by fragmentation in a Waring Blendor for 20 seconds followed by homogenisation in a Potter homogeniser. After centrifugation at 12,100g for 10 minutes the supernatant fraction was recovered. Extracts prepared in this way contained between 0.7-0.9mg protein/ml.

2.4.2 Plant Extracts.

In most cases plant tissue was quick-frozen in liquid nitrogen, ground to a fine powder using a pestle and mortar and suspended in buffer.

The use of liquid nitrogen in preparing plant extracts has been previously reported by Boey *et al.*, (1976) when investigating rhodanese in cassava and by Myers and Fry (1978) when investigating FHL in *Sorghum* sp.

For seedlings of *Lupinus augustifolia, Glycine max, Phaseolus aureus,* mature leaves of *Brassica oleraceae* var. capitata and for the mesocarp of loquat fruits *(E japonica),* extracts were prepared by homogenisation with the extracting buffer in a Waring Blendor for 45 seconds. Berries of *Nandina domestica* were ground with a pestle and mortar with buffer.

The crude suspension, however prepared, was squeezed through cheese-cloth and the filtrate centrifuged at 12,100g for 10 minutes at $4^{o}C$. The resulting supernatant fraction (hereafter termed the plant extract) was used for enzyme assays.

Removal of Phenolic Compounds from Plant Extracts.

'Plants produce a variety of phenolic compounds, which often interfere seriously in the isolation of plant enzymes .....', (Loomis, 1969). Phenols react with proteins either by reversible hydrogen bonding or, irreversibly, as a result of their oxidation to quinones followed by covalent condensations of these quinones with certain functional groups on the protein molecules. Both reactions may result in the precipitation and/or inactivation of enzymes.

Phenol oxidation may be either enzymatic, involving phenol oxidases or peroxidases, or it may be spontaneous. The spontaneous reaction is faster under alkaline conditions when the phenolic compounds are ionized. The oxidation of phenolic compounds commonly results in a "browning" of the plant extract due to the formation of melanin which may lead to the formation of insoluble melano proteins.

The most common means of removing phenols from plant extracts is to add a complexing agent such as polyvinylpyrrolidone (PVP). PVP contains

—CO—N< groups that are analogous to the peptide bonds of proteins and which form stable H-bonded complexes with phenolic -OH groups. Another phenolic complexing agent more recently developed is Amberlite XAD-4, a styrene-divinylbenzene polymer (Loomis *et al.*, 1979). This polymer adsorbs phenols primarily by hydrophobic interactions.

It was a common observation, during the preparation of extracts from light grown plants, that the extract became discoloured probably as a result of the oxidation of phenolic compounds. A similar observation was not observed in extracts of etiolated seedlings. Therefore, because of the undesirable effects of phenolic compounds, Amberlite XAD-4 resin was incorporated into the extraction procedure for the majority of light grown plants. In addition, these extracts were also passed through a Sephadex G-25 column (Pharmacia PD-10 column) after centrifugation at 12,100g for 10 minutes at 4^{o}C. Further details are given in Sections 2.7.1 & 2.7.2.

2.5 PROTEIN DETERMINATION

Protein in extracts of higher plants and in those of *G. sorghi* was determined by the method of Lowry *et al.*, (1951). Plant extracts were passed through a G-25 column (Pharmacia PD-10 column) and the protein fraction collected before determination. Bovine serum albumin was used as a protein standard.

The relative protein concentration in bacterial cell-free extracts was estimated by recording their extinction at 280nm.

2.6 MEASUREMENTS OF DRY WEIGHTS OF BACTERIA

The bacterial suspension (10mls) was centrifuged at 12,100g for 10 minutes and the supernatant fraction discarded. The cells were resususpended in approximately 10mls D.W., recentrifuged as before, the

supernatant fraction again discarded and the cells finally resuspended in D.W. to 10mls.

Aluminium foil boats (3cm in diameter and approximately 2cm deep) were weighed to ± 0.01mg. The washed bacterial suspension (1.0ml) was transferred to a foil boat and placed in an oven at approximately 105°C for 2 hours after which time the boat was weighed again. The dry weight of a bacterial suspension was always determined in duplicate.

2.7 ENZYME ASSAYS

2.7.1 The β-Cyanoalanine Synthase Assay

The β-cyanoalanine synthase assay was based on the formation of methylene blue from the H_2S produced by the enzyme reaction (Blumenthal *et al.*, (1968):

$$HSCH_2CHNH_2CO_2H + HCN \longrightarrow NCCH_2CHNH_2CO_2H + H_2S$$

cysteine → β-cyanoalanine

In most cases every 1g fresh weight of plant material was extracted with 2.5mls 0.05M Tris-HCl buffer (pH 8.5). For samples of *N. domestica*, the extracting buffer was 0.2M Tris-HCl (pH 8.5) (2.5mls : 1 g.f.w.) containing Amberlite XAD-4 resin (2 : 1 w/w resin : plant material), and the supernatant fraction, after centrifugation, was passed through a Sephadex G-25 column (Pharmacia PD-10 column) and the protein fraction collected for assay. Samples of mature loquat mesocarp were extracted with 0.2M Tris-HCl (pH 8.9). The pH and/or molarity of the extracting buffer was increased for samples of *N. domestica* and loquat mesocarp due to the acidic nature of these plant tissues.

The conditions for the enzyme assay itself were the same as those described by Blumenthal *et al.*, 1968. The substrates solutions were

prepared immediately prior to use. Thus, NaCN and L-CYS were dissolved separately in 0.1M Tris HCl buffer (pH 8.5) to give concentrations of 0.05M and 0.01M respectively and the pH of each solution was adjusted to 8.5. For the assay involving loquat mesocarp, however, the substrates were dissolved in 0.2M Tris-HCl (pH 8.5). The plant extract and substrate solutions were equilibrated separately to 30°C for 5 minutes after which time 0.5mls buffered NaCN solution was added to 1.0ml of plant extract followed by 0.5ml buffered L-CYS solution. The reaction mixture was incubated at 30°C in a glass bottle with a screw top for 10 minutes (in most cases) or for 30 minutes (when the extract had only a low β-cyanoalanine synthase activity). Plant extracts having high β-cyanoalanine synthase activity were diluted with pre-cooled 0.05M Tris-HCl buffer (pH 8.5) prior to assay. The reaction was stopped and the methylene blue formed by the rapid addition of 0.5mls of N,N-dimethyl-p-phenylene diamine sulphate (0.02M in 7.2M HCl) followed by 0.5mls $FeCl_3$ (0.03M in 1.2M HCl), the screw top being replaced after each addition. Samples were kept in the dark for 20-25 minutes and then centrifuged at 1050g for 5 minutes to remove precipitated protein. The absorbance of the supernatant liquid was measured at 650nm.

The β-cyanoalanine synthase activity for each plant was determined after subtracting the reading given by the appropriate boiled control. For the boiled control, a sample of the plant extract was heated in a boiling water-bath for 10-15 minutes. When cool, the boiled plant extract was assayed in exactly the same way as the experimental sample.

The standard, $Na_2S.9H_2O$, was dissolved in 0.05M Tris-HCl buffer (pH 8.5). The calibration graph was almost linear between 0-0.8 O.D. units (which corresponded to 0-208 nmoles sulphide/standard reaction mixture).

2.7.2 The Rhodanese Assay

Rhodanese catalyses the following reaction:

$$S_2O_3^{2-} + CN^- \longrightarrow SO_3^{2-} + SCN^-$$

The assay was based on the formation of a coloured complex between thiocyanate and ferric ions according to the method of Sörbo (1953).

In most cases every 1g fresh weight of plant material was extracted with 2mls 0.05M Tris-HCl buffer (pH 8.5). Extracts of berries of *N. domestica* were prepared as previously described in section 2.7.1 except for the ratio of plant material to extracting buffer. For samples of mature loquat mesocarp the extracting buffer was 0.2M Tris-HCl (pH 8.9).

Substrate solutions were prepared immediately prior to use. Thus, KCN and $Na_2S_2O_3$ were dissolved separately in 0.1M Tris-HCl buffer (pH 8.5) to give concentrations of 0.125M each. The pH of each solution was adjusted to 8.5. For the assay of loquat mesocarp, substrates were dissolved in 0.2M Tris-HCl (pH 8.5).

The substrate solutions were equilibrated to 30°C for 5 minutes prior to the start of the assay but plant extracts were kept at 0-4°C at this stage. To 1ml of the buffered KCN solution was added 1ml of the buffered $Na_2S_2O_3$ solution. After mixing, 0.5ml of the plant extract was added. Reaction mixtures were incubated at 30°C for 0, 30, 60 and 90 minutes (except for assays involving *Sorghum bicolor* which were incubated for 0, 5, 10 and 15 minutes only).

After the appropriate incubation time the reaction was stopped and the colour developed by the addition of 0.5mls of formaldehyde (37% w/v) followed by 2.5mls of ferric nitrate reagent. This reagent was composed of $Fe(NO_3)_3.9H_2O$, 50g; HNO_3 S.G. 1.40, 525mls; D.W. to 2l (Chew and Boey, 1972). The samples were immediately centrifuged at 1050g for 5 minutes

and the absorbance of the supernatant liquid recorded at 460nm. The rhodanese activity for each plant was determined after subtracting the reading given by the appropriate boiled control. The plant extract was boiled for 10-15 minutes and after cooling was treated in exactly the same way as the experimental sample. Rhodanese activity in the majority of plant extracts was also investigated at approximately pH 10.4. The substrates were then dissolved in 0.1M sodium borate/NaOH buffer (pH 10.4) and the pH adjusted to 10.4 immediately prior to use. The plant extracts used were the same as those prepared for the pH 8.5 assays. In all other respects the assays were performed as previously described.

The standard, NaSCN, dissolved in D.W., gave a linear calibration between 0-1.1 O.D. units (which corresponded to approximately 0-1.85 µmoles thiocyanate/standard reaction mixture).

There are several reasons for the use of formaldehyde in terminating the rhodanese reaction before determining the thiocyanate produced, by the Sörbo (1953) method:

(i) formaldehyde inactivates the enzyme

(ii) excess thiosulphate in the reaction mixture is decomposed by the acid in the ferric nitrate reagent resulting in the precipitation of sulphur; this is prevented in the presence of formaldehyde (Westley, 1973).

(iii) ferric ions complex with excess thiosulphate in the reaction mixture giving a blue colour which rapidly fades. Formaldehyde prevents the formation of this blue ferric-thiosulphate complex (Sörbo, 1953).

(iv) the iron-thiocyanate complex is unstable (possibly due to a photo-catalysed degradation reaction), but in the presence of formaldehyde its colour is stated to be completely stable for at least 1 hour

(Sörbo, 1953). However, absorbance readings in this investigation were taken as soon as possible after the addition of the ferric nitrate reagent since fading often began as early as 30 minutes.

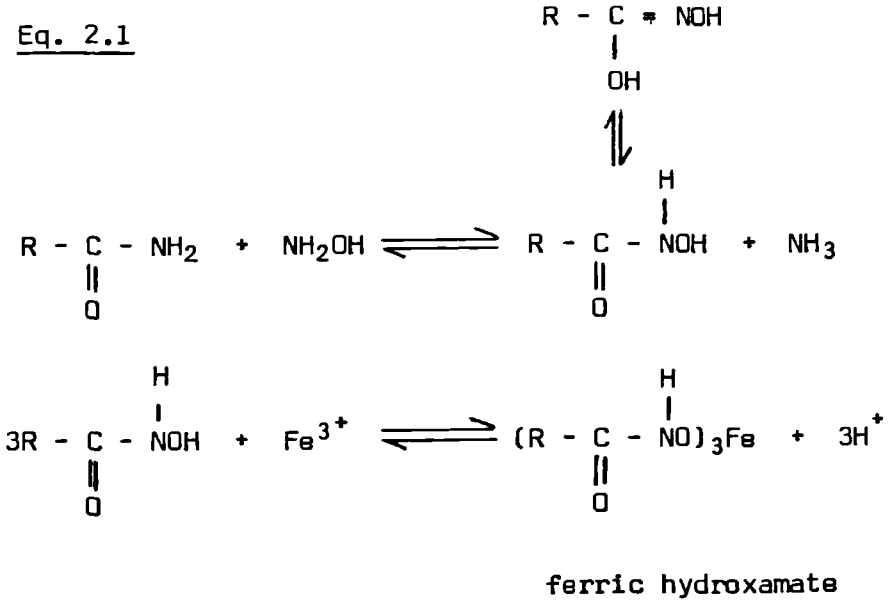
(v) formaldehyde removes excess cyanide (Westley, 1973).

The use of formaldehyde in terminating the rhodanese assay will be referred to again in Section 3.1.2.

2.7.3 The Formamide Hydro-lyase Assay

The FHL assay was based on the reaction between formamide and hydroxylamine (Fry and Millar, 1972; Snell and Snell, 1954). In detail, hydroxylamine reacts with certain carboxylic acid derivatives, e.g. esters, anhydrides, imides and amides, to form hydroxamic acids which undergo a colour reaction with ferric ions. The reaction with amides is schematically shown in Eq. 2.1.

Eq. 2.1



(Adapted from Soloway and Lipschitz, 1952)

In previous work on the FHL activity in the fungi *Stemphylium loti* (Rissler and Millar, 1977) and *Gloeocercospora sorghi* (Fry and Evans, 1977), the extracting buffer used was 0.05M Tris-HCl (pH 8.0). Thus,

unless stated otherwise, this buffer was also used for the preparation of plant extracts; 2mls of the buffer was used for every 1g fresh weight of plant material.

The substrate solution was prepared immediately prior to use. Sodium cyanide was dissolved in 0.05M Tris-HCl buffer (pH 8.0) to give an 0.1M solution which was adjusted to pH 8.0. The plant extract (1.0ml) was mixed with 1.0ml of the buffered NaCN solution and incubated at 25°C for 2 hours. This procedure was the same as that described previously by Rissler & Millar (1977) to determine FHL activity in *S. loti* and by Fry and Evans (1977) for *G. sorghi*.

For formamide analysis, 1.0ml of the reaction mixture was mixed with 2.0mls of freshly prepared alkaline hydroxylamine reagent (2.0M hydroxylamine hydrochloride/3.5M NaOH (1:1 v/v)). The sample was then heated at 60°C for 10 minutes and allowed to cool to room temperature before adding 1.0ml 4M HCl followed by 1.0ml of ferric chloride (0.74M) in HCl (0.07M). The sample was then usually centrifuged at 1050g for 3-4 minutes before recording the absorbance at 540nm. The absorbance was recorded immediately after centrifugation since the colour intensity of the ferric hydroxamate complex faded with time.

In addition to the experimental reaction mixtures, boiled extract controls, no substrate controls and no "enzyme" controls were also incubated for 2 hours and analysed for formamide production.

For the amide standard, the hydroxamate assay was applied to formamide (3.33µmoles/ml in 0.05M Tris-HCl, pH 8.0) and to a reagent blank (R.B.) containing buffer instead of formamide. Standards and R.Bs were included in every experiment, in duplicate; the average absorbance for 28 samples on 14 different occassions was:

	A540nm	S.D.
R.B.	0.054	0.019
Standard	0.544	0.075

Other experiments involving the hydroxamate reaction (used under different conditions) will be described in Section 5.1.1 which deals with the development of the nitrilase and amidase assays.

Shirai (1978) reported that loquat mesocarp converted HCN to ammonia. Thus, after the 2 hours incubation, in addition to formamide, reaction mixtureswere also analysed for ammonia production by nesslerisation. The reaction mixture (1.0ml) was placed in the outer-chamber of a 25ml conical flask having a centre-well containing 0.5mls of 0.5M H_2SO_4. To the outer-chamber was added, quickly, 1.0ml of a saturated solution of K_2CO_3. The flask was immediately sealed with parafilm and covered by a rubber cap. The flask was shaken at 30°C for 2 hours. The H_2SO_4 was then removed and the centre-well rinsed with 0.5mls 0.05M H_2SO_4. To the combined acid fractions (1.0ml) was added 5mls of Nesslers' reagent (Sigma ammonia colour reagent diluted 10-fold with D.D.W.). After 15 minutes the absorbance at 490 or 420nm was recorded.

For the ammonia standard, this microdiffusion procedure was applied to NH_4Cl (2.8μmoles/ml) in 0.05M Tris-HCl buffer (pH 8.0) and to a R.B. containing no NH_4Cl. Standards and RBs were included in every experiment, in duplicate; the average absorbance for 32 samples on 16 different occassions was:

	A_{490nm}	S.D
R.B.	0.013	0.007
Standard	0.451	0.060

Other experiments involving the analysis of ammonia with Nesslers' reagent (under modified conditions) will be described in Section 2.7.4. which deals with the nitrilase and amidase assays.

2.7.4. The Nitrilase and Amidase Assays.

Cells of the *Rhodococcus* sp. were usually grown in 5ℓ batch culture

(2.2.3) and harvested with a Sharples continuous centrifuge driven at 16,000-24,000 r.p.m. Cells were resuspended in 0.1M sodium phosphate buffer (pH 7.0) to 100mls. Thus the resulting cell suspension was 50 times as concentrated as the original culture. This concentrated cell suspension typically contained 10mg dry wt. of cells/ml (when grown in m.m. containing 0.25% v/v acetonitrile only).

Amidase activity was routinely assayed by determining the formation of ammonia from the hydrolysis of acetamide. The nitrilase activity was also assayed by the formation of ammonia, in this case from acetonitrile, by coupling the nitrilase activity to the amidase activity.

These activities were assayed using either intact cell suspensions or cell-free extracts prepared by sonication.

Unless stated otherwise, the buffer used throughout was 0.1M sodium phosphate (pH 7.0).

Modification 1.

In the earlier work, nitrilase and amidase activities were measured using the following reaction mixture: cell suspension or extract (1.0ml); buffer (3.0mls); 0.122M acetonitrile or acetamide in D.W. (1.0ml). In cases of high nitrilase or amidase activity the cell suspension/extract was diluted with buffer prior to assay. Unless stated otherwise, the cell suspension or extract was mixed with the buffer and equilibrated at 25°C for 3-5 minutes. The substrate solution, also at 25°C, was added and the complete mixture incubated at this temperature.

Modification 2.

In later work, reaction mixtures had the following composition: cell suspension or extract (2.0mls) and 2.0mls of either 0.1M acetonitrile or

acetamide in buffer. Again, when necessary, the cell suspension or extract was diluted with buffer prior to assay. The cell suspension or extract and the substrate solution were equilibrated separately to 25°C for 3 minutes and then mixed and incubated at this temperature.

At the end of the incubation period the formation of ammonia was determined by one of two methods,both involving the use of Nesslers' reagent.

Direct method.

In this method the Nesslers' reagent was added directly to the sample. The Nesslers' reagent was prepared as described by Burris (1972). Thus, light pieces of gum ghatti (0.825g) were ground to a powder with a pestle and mortar and refluxed with 375mls D.D.W. until it had dissolved (approximately 12 hours). Potassium iodide (2.0g) and mercuric iodide (2.0g), dissolved in 12.5mls D.D.W. were then added to the gum ghatti solution. The reagent was diluted to 500mls with D.D.W. and filtered.

The sample (1.0ml) was mixed with 1.0ml D.W. and then 2.0mls of Nesslers' reagent was immediately added followed by 3.0mls 2M NaOH. The absorbance of the mixture was recorded at 490nm exactly 5minutes later. When cell suspensions or "particulate" fractions were assayed however, after incubation the reaction mixture was centrifuged at 2,500g for exactly 5 minutes and the supernatant liquid taken for nesslerisation.

The standard, NH_4Cl, dissolved in 0.1M sodium phosphate buffer (pH 7.0), gave a linear calibration between 0 - 0.63 O.D. units (which corresponded to 0 - 3.75µmoles NH_3/ml sample).

Indirect method.

This method involved microdiffusion of the sample prior to determining the ammonia concentration.

Microdiffusion was carried out by a modification of the technique described by Etherington & Morrey (1967). Instead of KOH pellets however,

a saturated solution of K_2CO_3 was chosen as the alkali component to release ammonia from the sample.

Glass vials of approximately 20mls capacity, fitted with plastic caps, were used for microdiffusion, Fig. 2.2. A square of nylon material (previously washed in 1M sulphuric acid, rinsed in D.W. and dried) was placed in the cap and 3 drops of 1M sulphuric acid added to the nylon. The sample to be microdiffused (1.0ml) and the alkali component (1.0ml) were added to the vial. The sample was microdiffused for approximately 2 hours at room temperature in either an angled turntable or a linear shaker to promote microdiffusion. The cap was then transferred to a similar vial containing 10mls diluted Nesslers' reagent (BDH); reagent diluted 1 : 19 with D.D.W. The vial was shaken vigorously so as to dislodge the nylon square and the absorbance at 420nm recorded exactly 15 minutes later.

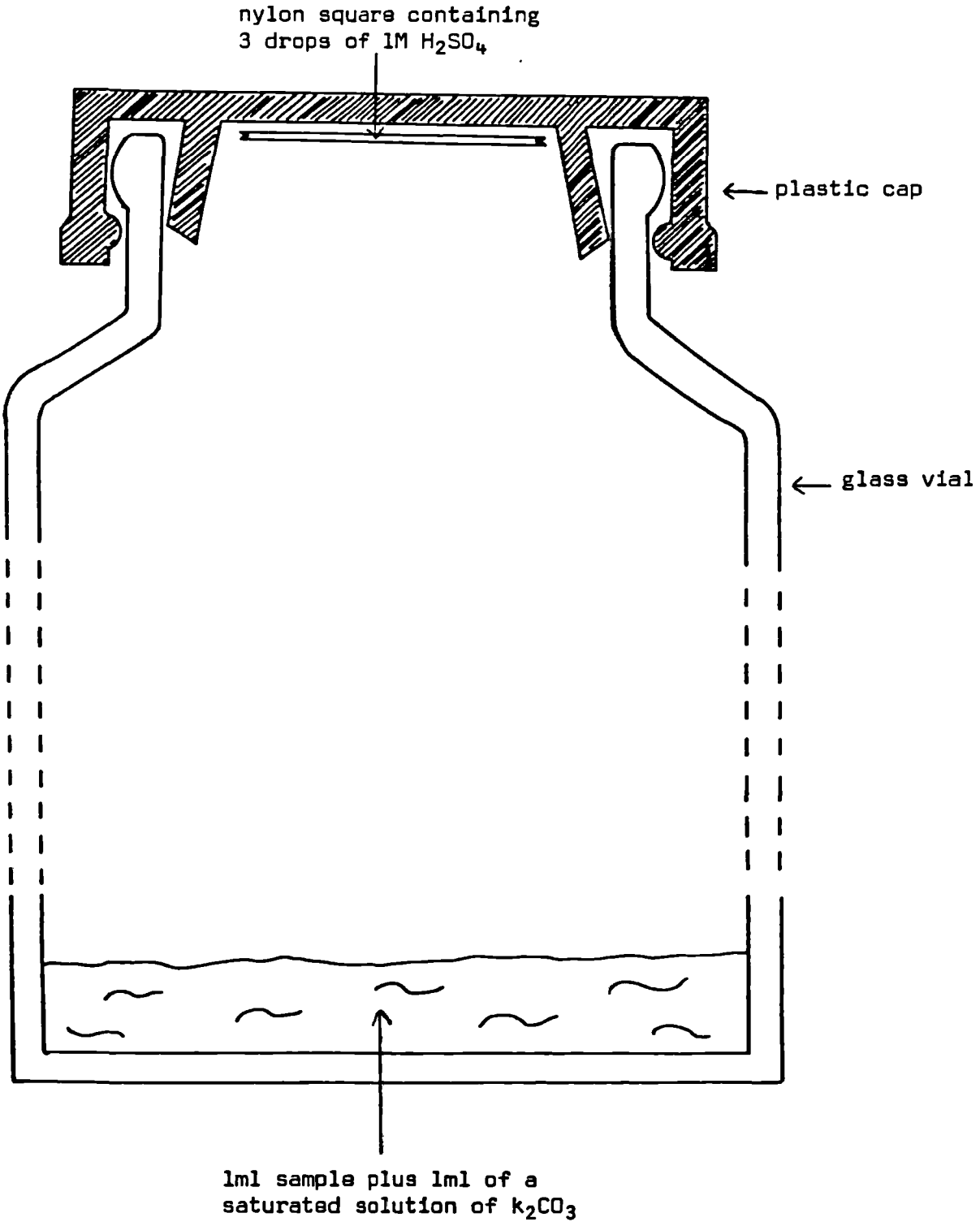
More experimental details about this method which were developed especially for the nitrilase and amidase assays are given in Section 7.1.1. and 7.1.2.

2.7.5. The Acyl-transferase Assay.

The *Rhodococcus* sp. was grown in 5l of m.m. containing acetonitrile (0.25% v/v) as the sole C and N source and then harvested and resuspended as described in Section 2.7.4. The transferase activity was measured by determining the amount of acethydroxamate formed from hydroxylamine and acetamide using intact cells of the bacterium according to the method of Brammar & Clarke (1964).

The substrate mixture was composed of equal volumes of (i) acetamide (0.4M in D.W.), (ii) hydroxylamine - HCl (2.0M in D.W., freshly prepared and neutralised) and (iii) 0.1M sodium phosphate buffer (pH 7.0). Bacterial suspension (0.25ml) was added to 0.75ml of the substrate mixture, both of

FIGURE 2.2. Vial for the Microdiffusion of Ammonia



which had been equilibrated to 25°C for 3 minutes. After 10 minutes incubation at this temperature the reaction was stopped by adding 2mls of a solution of $FeCl_3$ (anhydrous, 6% w/v in 0.23M HCl). The absorbance at 500nm was recorded immediately. The transferase activity was determined after substracting the reading given by a boiled control.

The standard, acethydroxamate, dissolved in 0.05M sodium phosphate buffer (pH 7.0), gave a linear calibration between 0 - 1.0 O.D. units (which corresponded to 0 - 2.7µmoles acethydroxamate/ml of sample).

2.8. PYRUVATE DETERMINATIONS.

Pyruvate production by extracts of *Brassica* seedlings (see Section 3.2) was determined by the method of Friedmann & Haugen (1943). Thus to 2mls of reaction mixture was added 3mls cold 10% (w/v) trichloroacetic acid. After 15 minutes the acidified mixture was centrifuged at 1050g for 5 minutes. An aliquot of the supernatant fraction (varying from 0.5 - 3.0mls) was made up to 3mls with 10% (w/v) T.C.A. Next, 1ml of 2,4-dinitrophenol (0.1% w/v in 2M HCl) was added followed 5 minutes later by 5mls 2.5M NaOH. The absorbance was recorded at 435nm 10 minutes later.

2.9. DETERMINATION OF HCN POTENTIALS.

Plant material (approximately 0.5g) was frozen in liquid nitrogen, ground to a fine powder, and placed in the outer chamber of a 50ml conical flask fitted with a centre-well. Into this centre-well was placed 1.0ml of 1M NaOH. Finally into the outer chamber was added 2 - 3 mls of an enzyme mixture capable of hydrolysing the known cyanogenic glycosides and having the following composition: 50mg commercial emulsin (Sigma), 5.0mls of a linamarase preparation* obtained from flax seedlings according to the

*The solution of linamarase was kindly prepared by J. Dunn, Department of Biochemistry and Biophysics, U.C.D.

method of Coop (1940) and 100mls 0.1M sodium phosphate buffer (pH 6.8). The flasks were sealed and shaken overnight at 37°C. The NaOH was removed and diluted to 2mls with 0.1M NaOH. Aliquots of this 2ml sample were taken and made up to 1ml with 0.1M NaOH.

The HCN concentration was determined colorimetrically by a modified method of Lambert *et al.*, (1975). After acidification of a 1.0ml aliquot of the cyanide sample with 0.5mls acetic acid (1M), 5.0mls of the succinimide-*N*-chlorosuccinimide reagent was added followed by 1.0ml of the barbituric acid-pyridine reagent. These reagents were composed of 0.6g succinimide, 0.06g N-chlorosuccinimide, D.W. to 1ℓ and 0.9g barbituric acid, 4.5mls pyridine, D.W. to 50mls, respectively. The absorbance of 580nm was recorded after 10 minutes at room temperature.

The standard, sodium cyanide, dissolved in 0.1M NaOH gave a linear calibration between 0 - 1.0 O.D. units (which corresponded to approximately 0 - 0.076µmoles inorganic cyanide/ml sample).

CHAPTER 3

THE METABOLISM OF HYDROGEN CYANIDE BY HIGHER PLANTS.

The research for the results presented in this Chapter was carried out in the Department of Biochemistry and Biophysics, University of California, Davis, Ca 95616, U.S.A.

This Chapter reports the investigation into the occurrence and distribution of HCN metabolising enzymes in a variety of higher plants, including both cyanogenic and non-cyanogenic species. This information in turn is compared with the HCN potential of each plant studied. The HCN potential is a measure of the concentration of cyanogenic glycosides in the plant which, upon degradation, leads to the release of HCN.

The enzymes selected for investigation were β-cyanoalanine synthase (EC 4.4.1.9), rhodanese (EC 2.8.1.1) and formamide hydrolyase (EC 4.2.1.66). Initially, however, a few experiments on the enzyme assays themselves were carried out.

3.1. PRELIMINARY INVESTIGATIONS INTO THE ENZYME ASSAYS.

3.1.1. The β-Cyanoalanine Synthase Assay: Effect of the pH and Composition of the Extracting Buffer.

Blue lupine (*Lupinus augustifolia*) seedlings showed β-cyanoalanine synthase activity when acetone powdered material was extracted with Tris buffer (pH 8.5-8.9) (Blumenthal *et al.*, 1968). Since the general method for preparing plant extracts, in this thesis, involved grinding tissue, frozen in liquid nitrogen, the extracting buffer first chosen was 0.01M sodium phosphate (pH 6.8). It was hoped that the low pH would reduce any interference from plant phenolics. However, extracts of blue lupine prepared in this way and assayed as described in Section 2.7.1. appeared to contain no β-cyanoalanine synthase activity. When the extracting buffer was changed to 0.05M Tris-HCl (pH 8.5), however, the enzyme activity was easily detectable.

Extracts of *Sorghum bicolor* (Linn.) moench were prepared in the same two buffers and assayed for β-cyanoalanine synthase. Activity was again greater after extraction with Tris buffer (Table 3.1). Extracts of the shoot and root in Tris buffer gave activities approximately 15 and 34 times (respectively) greater than the extracts prepared in phosphate buffer, calculated on a fresh weight basis. Enhanced β-cyanoalanine synthase activity for extracts prepared in the Tris buffer as opposed to the phosphate buffer was also noted for *Trifolium repens* and *Linumusitatissimum*. Therefore Tris buffer was always used subsequently when preparing extracts for the β-cyanoalanine synthase assay.

3.1.2. The Rhodanese Assay: the Spontaneous Reaction of Cyanide with Thiosulphate to form Thiocyanate at Different Temperatures and pH.

Rhodanese catalyses the conversion of cyanide and thiosulphate to thiocyanate and sulphite. However, the same reaction also proceeds non-enzymatically at least at steam-bath temperatures (Sörbo, 1957).

Chew and Boey (1972) stated that for the rhodanese in cassava, "optimal activity was found at high pH (10.2-11.0) and temperature (57-59°C). These are precisely the conditions that would be expected to stimulate the chemical formation of thiocyanate. The control used by Chew and Boey to estimate rhodanese in a variety of plants involved mixing the crude extract with formaldehyde before the addition of the substrates. However, formaldehyde has been said to remove excess cyanide from solution (see Section 2.7.2.) so this control may underestimate the non-enzymatic reaction.

The present experiment was designed to investigate this possibility and to study the effect of temperature and pH on the non-enzymatic formation of thiocyanate. Three buffers were used:

(i) 0.1M sodium borate/HCl, pH 8.5
(ii) 0.1M sodium borate/HCl, pH 9.2
(iii) 0.1M sodium borate/NaOH, pH 10.4

TABLE 3.1

β-Cyanoalanine Synthase Activity in *S. bicolor* (Linn.) moench:

Effect of the Composition of the Extracting Buffer

Extracting buffer	Tissue	A 650nm
0.01M sodium phosphate (pH 6.8)	shoots	0.625 ± 0.020
	roots	0.023 ± 0.001
0.05M Tris-HCl (pH 8.5)	shoots	9.600 ± 0.080
	roots	0.771 ± 0.015

After grinding the frozen tissue, 2.5mls of phosphate or Tris-HCl buffer was added per g.f.w. β-Cyanoalanine synthase activity was determined as described in Section 2.7.1. Reaction mixtures were incubated for 10 minutes. The A650nm values given are the difference between the experimental and boiled controls multiplied by the factor by which the extract was diluted prior to assay.

These were each incubated with cyanide and thiosulphate at 25°C, 35°C and 55°C, both in the presence and absence of formaldehyde (Table 3.2).

Fig. 3.1. shows that the rate of chemical formation of thiocyanate increases with increasing pH and temperature. Table 3.2 shows that there was very little formation of thiocyanate in any of the samples to which formaldehyde was added at the beginning of the incubation period although even here the reaction rate was stimulated slightly by increasing temperature. Although the non-enzymatic reaction is fairly slow at pH 8.5 and 25°C it is much faster at pH 10.4 and 55°C; here thiocyanate is being formed approximately 110 nmoles/reaction mixture/minute faster in the absence of formaldehyde.

The control used by Chew and Boey therefore appears not to take into account the non-enzymatic formation of thiocyanate and so boiled extract controls were chosen for all subsequent rhodanese assays described in this thesis.

3.1.3. The Rhodanese Assay: Effect of Thiosulphate and BSA in the Extracting buffer on the Activity and Stability of *Sorghum* Rhodanese.

It has previously been reported that $Na_2S_2O_3$ and BSA (bovine serum albumin) stabilize rhodanese and prevent it from being inactivated when diluted with buffer (Sörbo, 1953). Thus, the effect of incorporating these two components, at the recommended concentrations, into the extracting buffer was studied for the rhodanese activity of *S. bicolor* (Linn.) moench.

Extracts of 3-day old etiolated shoots were prepared by grinding the frozen tissue and resuspending 1 g.f.w. in 2mls of either:

(i) 0.01M sodium phosphate buffer (pH 6.8)

(ii) 0.01M sodium phosphate buffer (pH 6.8) containing 0.025% (w/v) BSA and 0.0125M $Na_2S_2O_3$.

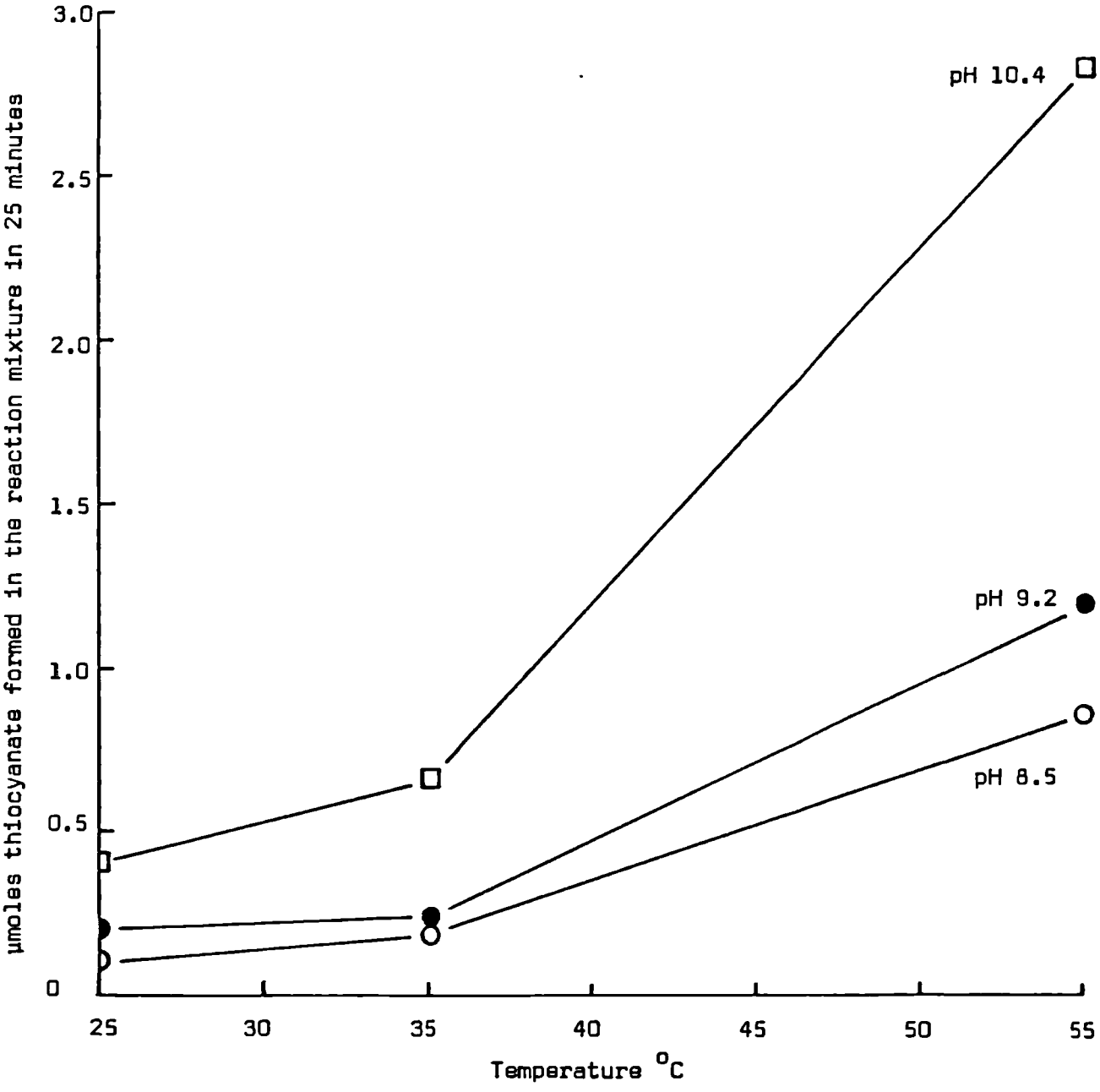
TABLE 3.2.

The Spontaneous Formation of Thiocyanate from Cyanide and Thiosulphate: Variation with Temperature and pH

pH	Temperature ^{o}C	Sample (+ formaldehyde)	A_{460nm}
8.5	25	-	0.060
	"	+	0.029
	35	-	0.105
	"	+	0.038
	55	-	0.503
	"	+	0.060
9.2	25	-	0.120
	"	+	0.040
	35	-	0.137
	"	+	0.041
	55	-	0.701
	"	+	0.043
10.4	25	-	0.234
	"	+	0.038
	35	-	0.385
	"	+	0.055
	55	-	1.660
	"	+	0.057

The composition of the reaction mixture and the method for determining the formation of thiocyanate was essentially as described in Section 2.7.2, However, the two reactants, KCN and $Na_2S_2O_3$, were dissolved separately in each of the buffers mentioned in the text and 0.01M sodium phosphate buffer replaced the plant extract. For samples incubated in the presence of formaldehyde, this was added immediately after the phosphate buffer. All samples were incubated for 25 minutes.

FIGURE 3.1. The Spontaneous Formation of Thiocyanate From Cyanide and Thiosulphate : Variation with Temperature and pH.



Experimental details are given in Table 3.2. The volume of each reaction mixture was 2.5mls.

The resulting extracts, after centrifugation, were assayed for rhodanese activity immediately and again after being kept in an ice-bath for 2 hours.

Table 3.3. shows that there was more rhodanese activity in the plant extract containing no BSA and $Na_2S_2O_3$. Furthermore, these supplements did not stabilize the enzyme; the loss of activity over 2 hours was 17% for the extract in buffer only, compared to 19% in buffer plus BSA and $Na_2S_2O_3$. Moreover, the supplements increased the values of the no substrate controls by approximately 2.3 times. This suggested enzymatic and/or non-enzymatic formation of thiocyanate from the added $Na_2S_2O_3$ and endogenous cyanide (from the breakdown of dhurrin). Non-enzymatic formation of thiocyanate would certainly be extensive if any extract of a cyanogenic plant containing added $Na_2S_2O_3$ was boiled. This was most undesirable since boiled controls were considered important in the rhodanese assay to quantify the non-enzymatic formation of thiocyanate. Therefore, in future experiments, the crushed plant tissue was extracted with buffer only.

3.1.4 The Rhodanese Assay: Effect of Temperature and pH on the Activity in Extracts of *S.bicolor* (Linn.) moench.

The pH and temperature optima for rhodanese activity in crude extracts of cassava leaves were reported to be pH 10.2 - 11.0 and 57-59°C (Chew and Boey, 1972) whereas for the purified enzyme from cabbage leaves optimum activity was at pH 8.0 - 8.5 and 50 - 55°C (Tomati *et al.*, 1974). The effect of these two variables on rhodanese activity in extracts of *Sorghum* was therefore investigated.

Fig. 3.2 shows that after correcting for non-enzymatic formation of thiocyanate, activity increased with increasing temperature at each pH. Also, the rhodanese activity was almost always higher the more basic the pH, at each temperature. However, at pH 10.4 increasing the temperature stimulated activity less than at pH 8.5. This may be due to a faster

TABLE 3.3.

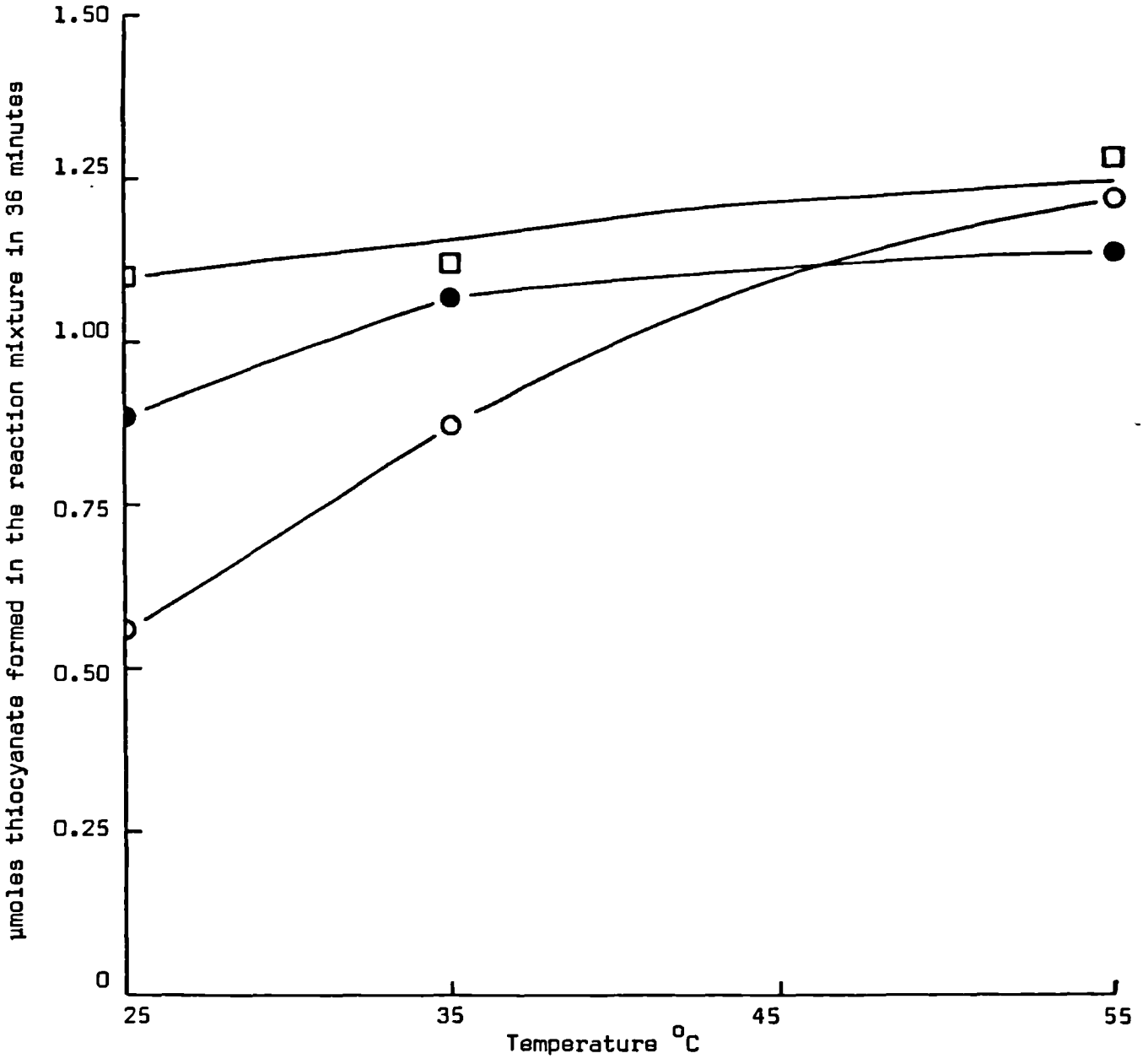
Rhodanese Activity of *S. bicolor* (Linn.) moench Extracts:

The Effect of "Stabilizers" in the Extracting Buffer .

Composition of extracting buffer	Assay	Rate of thiocyanate formation (µmoles in the reaction mixture in 5 mins.).	
		1st assay	2nd assay
0.01M sodium phosphate (pH 6.8)	Experimental	0.775 ± 0.003	0.654 ± 0.010
	No substrates control	0.055 ± 0.005	0.055 ± 0.002
	Experimental-No substrates control	0.720 ± 0.008	0.599 ± 0.012
0.01M sodium phosphate (pH 6.8) + BSA & $Na_2S_2O_3$	Experimental	0.616 ± 0.009	0.538 ± 0.009
	No substrates control	0.119 ± 0.000	0.136 ± 0.012
	Experimental-No substrates control	0.497 ± 0.009	0.402 ± 0.021

Details of the rhodanese assay are given in section 2.7.2. The 1st assay was carried out immediately after preparing the extract whereas the 2nd assay was carried out 2 hours later. Results are the average of duplicate assays. Although the no substrate control for the extract in buffer only would not take into account the non-enzymatic formation of thiocyanate, from Table 3.2. it can be calculated that after a 5 minute incubation at 25°C and pH 8.5 only 0.021 µmoles thiocyanate would be formed spontaneously in the experimental reaction mixture. The corresponding value for the non-enzymatic formation of thiocyanate in the extract containing the "stabilizers" could not be calculated exactly from the available data due to the presence of $Na_2S_2O_3$. It is important to note however that the value for the experimental-no substrate control in the latter case may be slightly underestimated since some enzymatic formation of thiocyanate could have occurred in the no substrates control.

FIGURE 3.2. Rhodanese Activity in Extracts of *S. bicolor* (Linn.) moench : Effect of Temperature and pH.



Rhodanese activity in extracts of *S. bicolor* was measured at pH 8.5, (O-O); pH 9.2, (●-●) and pH 10.2 (□ - □). The plant extract was prepared as described in Section 2.4.2 using 0.01M sodium phosphate (pH 6.8) as the extracting buffer. Substrates, KCN and $Na_2S_2O_3$, were dissolved(separately) in the same 3 buffers as detailed in Section 3.1.2. The rhodanese assay was carried out as in Section 2.7.2 with the exception of the variable temperature and pH. Results were calculated after subtracting the appropriate boiled control from the experimental sample and are the average of duplicate assays. Due to the extent of non-enzymatic formation of thiocyanate at high temperatures and pH every sample was diluted x3 with D.D.W. prior to recording the A_{460}nm.

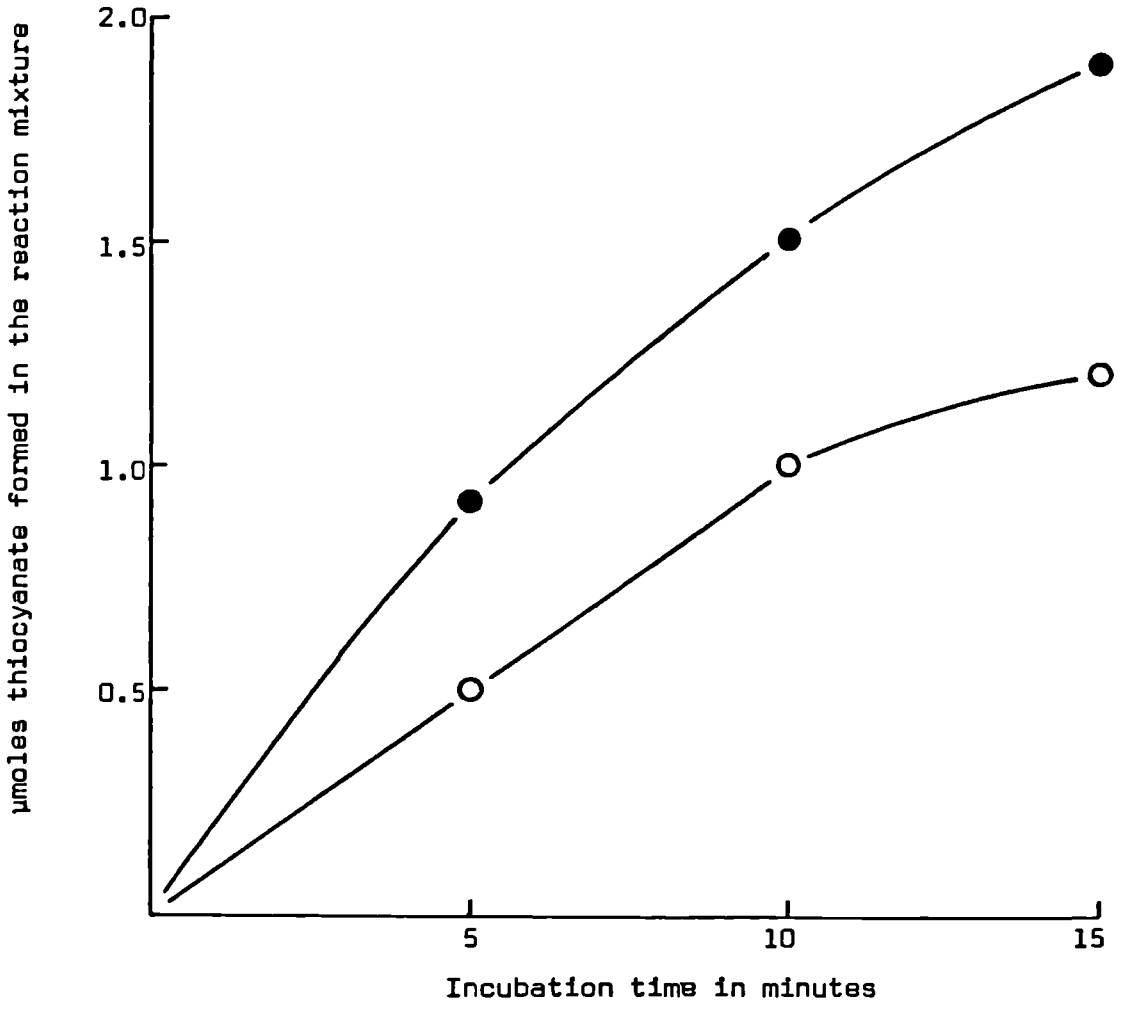
denaturation of the rhodanese enzyme at high pH.

Although the rhodanese activity in *Sorghum* shoots appeared to be greatest at pH 10.4 and 55°C, the absorbance for the boiled control was so high that it would always necessitate diluting samples prior to recording the optical density. Thus, if there was only a small difference in the amount of thiocyanate formed, between the experimental and boiled control samples, it would be difficult to detect due to the vast amount of thiocyanate formed non-enzymatically. It was therefore decided to do the rhodanese assays at pH 8.5 and 30°C, where there would be less non-enzymatic formation of thiocyanate, although with several plant species the assay was also carried out at pH 10.4 and 30°C.

3.1.5. The Rhodanese Assay: Effect of the pH and Composition of the Extracting Buffer.

After discovering the enormous effect the nature and pH of the extracting buffer had upon the activity of β-cyanoalanine synthase in some plant extracts, similar investigations were made for rhodanese activity in 3-day old etiolated *Sorghum* shoots. The extracts (prepared as in Section 3.1.1, except only 2mls extracting buffer was added/g.f.w. tissue) were assayed for rhodanese activity at pH 8.5 and 30°C. Fig.3.3 shows that it was better to use the Tris buffer (pH 8.5) rather than the phosphate buffer (pH 6.8) for resuspending the plant homogenate, at least in the case of *Sorghum* shoots. However, the effect of the nature of the extracting buffer upon rhodanese activity was not so great as that for β-cyanoalanine synthase in *Sorghum*. Therefore in future experiments, plant extracts used for the determination of rhodanese were usually prepared in 0.05M Tris-HCl (PH 8.5) with exceptions noted in Section 2.7.2.

FIGURE 3.3. Rhodanese Activity in *S. bicolor* (Linn.) moench : Effect of the Composition of the Extracting Buffer.



Rhodanese activity was measured for plant extracts in 0.01M sodium phosphate, pH 6.8, (O - O) or in 0.05M Tris-HCl, pH 8.5, (● - ●). The rhodanese assay was carried out as described in Section 2.7.2.

3.1.6. The Formamide Hydro-lyase Assay: Activity in *Gloeocercospora sorghi*.

The only plant extracts previously reported to contain FHL were those of the mesocarp of the loquat (*Eryobotrya japonica*) and of the Japanese apricot (*Prunus mume* Sieb et Zucc) (Shirai, 1978). Since loquat fruits were not in season when beginning this project it was decided to test the FHL assay using extracts of *G. sorghi*, a fungal plant pathogen known to possess FHL (Fry and Munch, 1975).

FHL activity was certainly detected. Extracts prepared from non-induced mycelia and those grown in the presence of KCN (see Section 2.2.3) had FHL activities of 0.136 $\pm$ 0.005 and 3.8 $\pm$ 0.2 µmoles formamide produced/mg protein/minute respectively. This confirmed that cyanide induces enzyme synthesis as first discovered by Fry and Munch (1975). The activity in extracts of induced *G. sorghi* was however only 9% of that originally reported by Fry and Evans (1977). Slightly different growth conditions and/or the exact stage of growth at which the culture was harvested may account for this difference.

Shirai (1978) reported that a partially purified enzyme preparation from the mesocarp of loquat produced formate from NaCN and that the crude extract formed ammonia from KCN. Therefore, in all future experiments aimed at investigating FHL activity in plants, reaction mixtures were analysed for both formamide and ammonia.

3.2. β-CYANOALANINE SYNTHASE ACTIVITY IN HIGHER PLANTS.

Values for β-cyanoalanine synthase activity in extracts of cyanogenic higher plants are shown in Table 3.4. It can be seen that the enzyme was present in every plant tested. Moreover, there was a general correlation between enzyme activity and HCN potential. The higher the HCN potential, in general, the higher the β-cyanoalanine synthase activity.

TABLE 3.4

β-Cyanoalanine Synthase Activity in Cyanogenic Plants.

Plant	Tissue and Growth Conditions	β-Cyanoalanine Synthase Activity	β-Cyanoalanine Synthase Activity	HCN Potential
		nmol H_2S/g.f.w. tissue/minute	nmol H_2S/mg protein/minute	µmol HCN/ g.f.w. tissue
Nandina domestica	Buds	1050 ± 42	113 ± 24	205 ± 37
	Berries	51 ± 2	10.0 ± 0.9	0.28 ± 0.04
Phaseolus lunatus	Leaves	869 ± 50	131 ± 11	27.5 ± 5.2
Sorghum bicolor moench	3-day seedlings:			
	shoots	600 ± 20	115 ± 5	38.3 ± 1.7
	roots	123 ± 1	63.7 ± 1.8	4.62 ± 0.23
Linum usitatissimum	6-day seedlings:			
	shoots	91.5 ± 0	19.8 ± 0.3	17.4 ± 1.5
	roots	26.5 ± 0	11.3 ± 0.1	15.6 ± 0.2
Lotus tenuis	9-day seedlings: whole seedlings	88.0 ± 0.3	31.9 ± 2.4	4.3 ± 0.7
Eschscholzia californica	5-day seedlings: whole seedlings	80.0 ± 0.6	26.6 ± 1.4	2.87 ± 0.01
Trifolium repens	5-day seedlings: whole seedlings	31.9 ± 0.3	13.8 ± 0.4	0.13 ± 0.01
Vicia sativa	10-day seedlings: whole seedlings	28.1 ± 0.3	5.0 ± 0.6	0.05 ± 0.02
Eryobotrya japonica	Mature mesocarp	3.3 ± 0.1	1.7 ± 0.2	0.17 ± 0.02

β-Cyanoalanine synthase was assayed as described in Section 2.7.1.
Results are the average of duplicate assays.

Table 3.5 shows the results obtained for non-cyanogenic higher plants. The activity of β-cyanoalanine synthase in non-cyanogenic plants was found, in general, to be lower than that observed in cyanogenic plants. The notable exception was cabbage (*B.oleraceae* var. capitata), both in seedlings and mature leaves.

In the light of the results obtained for cabbage, explanations for the production of H_2S involving enzymes other than β-cyanoalanine synthase were considered. Several members of the family Cruciferae (including cabbage) have been shown to contain the enzyme cystine lyase (Mazelis *et al*, 1967); a pathway for the formation of H_2S involving this enzyme was considered possible (Fig. 3.4). In this scheme, cysteine is oxidised to cystine which is then converted to thiocysteine, pyruvate and ammonia by cystine lyase. The reactive product, thiocysteine, may then be chemically converted back to cystine in the presence of cysteine with the simultaneous release of H_2S. Another conceivable mechanism for forming H_2S is that the enzyme cyastathionine-γ-lyase (EC 4.4.1.1) could act directly on the substrate cysteine, (Fig. 3.4).

Since both reactions involve the formation of pyruvate, the production of this compound from cysteine was investigated. Table 3.6 shows that extracts of seedlings of both *B. oleraceae* var. capitata and *B.juncea* var. foliosa formed pyruvate in the presence of cysteine. However, this formation of pyruvate was inhibited by 12.5mM NaCN; this was the final concentration of cyanide in reaction mixtures used for β-cyanoalanine synthase assays. It is therefore likely that the H_2S production recorded for the *Brassica* species in Table 3.5 was really the result of β-cyanoalanine synthase activity.

3.3. RHODANESE ACTIVITY IN HIGHER PLANTS.

Each species listed in Tables 3.4 and 3.5 was examined for rhodanese.

TABLE 3.5

β-Cyanoalanine Synthase Activity in Non-Cyanogenic Plants.

Plant	Tissue and Growth Conditions	β-Cyanoalanine Synthase Activity	β Cyanoalanine Synthase Activity	HCN Potential
		nmol H_2S/g.f.w. tissue/minute	nmol H_2S/mg protein/minute	µmol HCN/ g.f.w. tissue
Lupinus augustifolia	10-day seedlings: whole seedlings	24.0 ± 0.6	5.0 ± 1.0	ND
Phaseolus aureus	5-day seedlings: whole seedlings	14.1 ± 0.3	2.7 ± 0.1	ND
Medicago sativa	5-day seedlings: whole seedlings	7.8 ± 0.3	2.2 ± 0.1	ND
Glycine max	4-day seedlings: whole seedlings	3.92 ± 0.04	0.101 ± 0.006	ND
Brassica oleraceae var. capitata	4-day seedlings: whole seedlings	75.6 ± 3.8	14.5 ± 1.1	ND
	mature leaves	130.6 ± 3.1	29.7 ± 2.5	ND
Brassica juncea var. foliosa	4-day seedlings: whole seedlings	25.0 ± 1.3	8.5 ± 1.0	ND

ND: not detected

Experimental details as for Table 3.4

FIGURE. 3.4. Possible pathways for H_2S production from cysteine involving the enzymes cystine lyase and cystathionine-γ-lyase

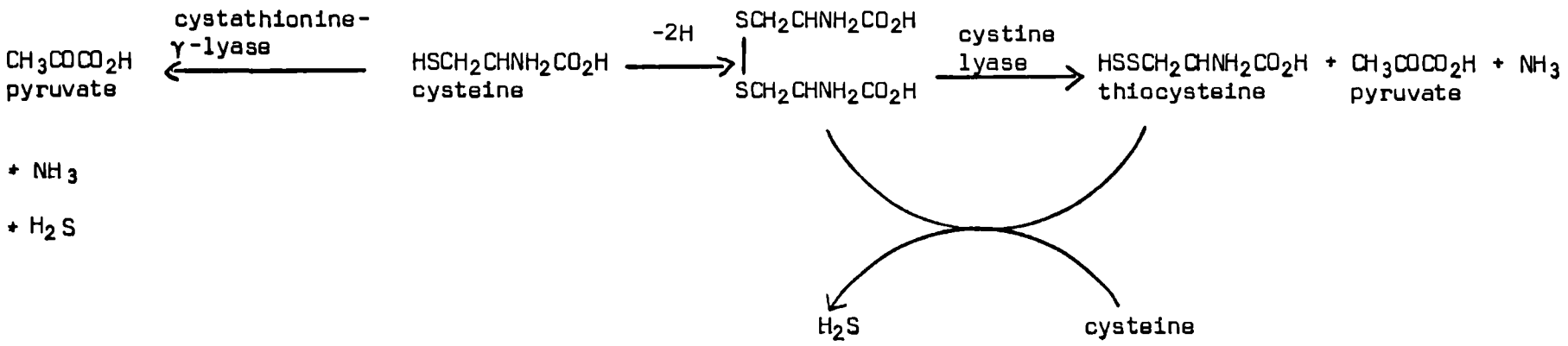


TABLE 3.6

Formation of Pyruvate from Cysteine by Extracts of *Brassica* Seedlings in the Presence and Absence of Cyanide

Brassica sp.	Assay	A435nm
B. oleraceae var. capitata	Experimental - HCN	0.543 ± 0.007
	Experimental + HCN	0.484 ± 0.004
	Boiled extract control + HCN	0.479 ± 0.001
	No substrate control	0.480 ± 0.001
B. juncea var. foliosa	Experimental - HCN	0.882 ± 0.003
	Experimental + HCN	0.679 ± 0.004
	Boiled extract control + HCN	0.695 ± 0.008
	No substrate control	0.773 ± 0.006

Growth and preparation of extracts of the two seedlings were as described for the β-cyanoalanine synthase assay. For the experimental-HCN assay, to 1ml of the extract at 30°C was added 0.5ml o.1M Tris-HCl buffer (pH 8.5) and 0.5ml 0.01M L-CYS in the same buffer, both at 30°C. The experimental + HCN reaction mixture was of similar composition except that the reaction mixture also contained 12.5mM cyanide. All reaction mixtures were incubated for 30 minutes at 30°C and pyruvate formation determined as described in Section 2.8.

activity. Table 3.7 lists those species for which rhodanese was detected. The results are for assays carried out at 30°C and pH 8.5. In addition extracts of 10-day old seedlings of etiolated common vetch (*V. sativa*) also appeared to give a marginally positive result for rhodanese activity (0.79 ± 0.03 nmoles thiocyanate/mg protein/minute). However, this result was suspect because experimental reaction mixtures remained slightly opaque even after centrifugation whereas for the boiled controls the supernatant fraction was clear.

When assayed at 30°C and pH 10.4, rhodanese activity in extracts of 3-day old seedlings of *S. bicolor* (Linn.) moench was greater than that at pH 8.5. Extracts of *V. sativa* also appeared to give a marginally positive result under these conditions but even less than that at pH 8.5. No activity was detected, however, for extracts of *E. californica* when assayed at pH 10.4. Extracts of *B. oleraceae* var. capitata and *B. juncea* var. foliosa were not tested for rhodanese activity at pH 10.4. It is interesting that the very high rhodanese activity of 3-day old etiolated shoots of *S. bicolor* is correlated with a high HCN potential.

Rhodanese activity can also be detected by the N-methylphenazonium-methosulphate mediated, sulphite reduction of 2,6-dichlorophenol-indophenol. The reduction can be followed spectrophotometrically by recording the decrease in absorption with time at 600 nm. This assay method was applied to extracts of *Sorghum* shoots to confirm the positive rhodanese results already obtained.

The method was followed exactly as described by Smith and Lascelles (1966) except that the concentration of DCPIP in the reaction mixture was reduced by 50%. The pH of reaction mixtures prepared in this way was 9.0. During the 6 minutes incubation period no reduction in A600nm was recorded for the boiled extract control, no substrate control and no enzyme control. For the experimental reaction mixture however, a reduction in A600nm

TABLE 3.7

Rhodanese Activity in Cyanogenic and Non-Cyanogenic Plants

Plant	Tissue and Growth Conditions	Rhodanese Activity	Rhodanese Activity[a]	HCN Potential
		nmol thiocyanate/ mg protein/minute	nmol thiocyanate/ g.f.w. tissue/ minute	µmol HCN / g.f.w. tissue
S. bicolor moench	3-day seedlings	252 ± 15	923	38.3 ± 1.7
E. californica	5-day seedlings: whole seedlings	2.49 ± 0.14	7.3	2.87 ± 0.01
B. oleracea var. capitata	4-day seedlings: whole seedlings	7.73 ± 0.04	24.2	ND
	mature leaves	1.73 ± 0.09	10.7	ND
B. juncea var. foliosa	4-day seedlings: whole seedlings	5.2 ± 0.3	9.7	ND

ND: not detected

Rhodanese was assayed as described in Section 2.7.2. Activity was determined at 30°C and pH 8.5. Results are the average of duplicate assays.

of 0.29 O.D. units was recorded over the 6 minute period. Taking the millimolar extinction coefficient of DCPIP at 600nm to be 19.1, the rhodanese activity in *Sorghum* resulted in a reduction of 37.6 nmoles DCPIP/g.f.w. tissue/minute as calculated from the initial rate (0.06 O.D. units/minute). Thus, rhodanese activity in *Sorghum* shoots was confirmed by this alternative assay.

Extracts of *L. tenuis*, for which rhodanese activity could not be detected by the method of Sörbo (1953) was also assayed by the DCPIP method. In agreement with the original assay, no rhodanese activity could be demonstrated.

3.4. FORMAMIDE HYDRO-LYASE ACTIVITY IN HIGHER PLANTS.

Formamide production from HCN was not observed in extracts of any of the higher plants listed in Tables 3.4 and 3.5. If HCN is hydrolysed in higher plants, formamide may not accumulate but be further hydrolysed to formic acid and ammonia. Formamide and ammonia were therefore determined simultaneously. However, no ammonia formation, above that of the boiled control, was detected for any of the plants listed.

Since FHL activity had been reported in the mesocarp tissue of the mature loquat fruit (Shirai, 1978), the hydrolysis of HCN to formamide and/or ammonia was investigated for different tissues of the loquat tree at different stages of development throughout the year. The tissues studied were green seeds which were present inside the decomposing fruit in October/November, unripe fruit in March when the exocarp was dark green and the endocarp light green, young leaves (April) and the endocarp of the mature fruit (June).

Most tissues were homogenised in a Waring Blendor but extracts of young leaves were prepared by grinding the frozen tissue in liquid nitrogen.

Except for the mature fruits, extracts were dialysed (4-12 hours with 1-2 buffer changes) against 0.05M Tris-HCl (pH 8.0) to remove small molecules from the extracts such as phenols, which interfered with the hydroxamate test.

The FHL assay, involving the determination of both formamide and ammonia, was carried out as described in Section 2.7.3. However, ammonia production was not measured for extracts of green seeds. All results were nominally negative in that experimental samples and the corresponding boiled extract controls gave similar results.

Shirai (1978) homogenised the loquat mesocarp in water before measuring FHL activity. Therefore, in this experiment, D.D.W. was used to prepare a similar extract. Again, however, after incubating the extract with HCN for 2 hours no enzymic formation of formamide or ammonia could be detected. The pH of the extract prepared in D.D.W. was measured and found to be very acidic, i.e. pH 3.5. Even upon addition of the buffered substrate the pH of the reaction mixtures did not rise above 4.8.

In view of the acidic nature of the extract in D.D.W. extracts were prepared in a variety of buffers and the pH recorded. The buffers were: (i) 0.1M Tris-HCl (pH 8.0); (ii) 0.2M Tris HCl (pH 8.0); (iii) 0.2M Tris-HCl (pH 8.5); (iv) 0.2M Tris-HCl (pH 8.9). The pH of these extracts were recorded as 3.9, 4.6, 7.0 and 7.7 respectively.

The extract at pH 7.7 was assayed for FHL activity as usual (by determining both formamide and ammonia production). Again the results for the edperimental samples and boiled extract controls were similar.

However, despite these negative results it was often noted that for the ammonia assay the experimental sample and the boiled extract control gave higher O.D. values than the summation of the no substrate and no enzyme controls. This was observed for extracts of mature loquat mesocarp, *Sorghum*

shoots, common vetch (*V. sativa*), mung bean (*P. aureus*) and alfalfa (*M. sativa*) seedlings and mature cabbage (*B. oleraceae*) leaves.

The situation was further investigated using extracts of the mature loquat mesocarp. A boiled extract control incubated without substrate gave a similar O.D. to that of the no substrate control. Thus the observed increase in O.D. of the boiled control plus substrate above that of the other controls was not due to the release of ammonia during boiling.

The results in Table 3.8 show that the extra ammonia present in the experimental samples and boiled extract (+ substrate) controls was actually formed during the 2 hour incubation period and not, for example, spontaneously during microdiffusion. Thus it appears that some plant extracts can catalyse a non-enzymic conversion of HCN to ammonia.

The next experiment was designed to determine whether 'non-enzymic' hydrolysis of HCN to ammonia was due to a high or low m.w. component in the loquat extract. A Pharmacia PD-10 (G-25) column was equilibrated with 0.2M Tris-HCl (pH 8.0) and 2.5mls extract (prepared as in the last experiment) was applied to the column. The column was eluted with similar buffer and, after discarding the void volume, the first 3.5 mls was collected (i.e. the high m.w. fraction) and assayed for ammonia production from HCN.

The results were negative (similar to the zero-time assays in Table 3.8) which indicated that the component in the extract which catalyses the conversion of HCN to ammonia did not have a high m.w., i.e. it was not a protein. However, the possibility remained that a heat stable enzyme was responsible but that the G-25 fractionation separated it from some essential cofactor. Alternatively, a heat stable enzyme might have been permanently bound to the column.

Therefore, in the next experiment the high m.w. fraction was again collected but the column was further eluted to collect the low m.w. fractions. Assays were carried out for each fraction separately and also for a

TABLE 3.8

Ammonia Formation with Time by Extracts of Mature Loquat Mesocarp Incubated with HCN

Assay	A490nm	
	Incubation time in hrs.	
	0	2
Experimental	0.049 ± 0.006	0.441 ± 0.030
Boiled extract (+ substrate)	0.042 ± 0.007	0.472 ± 0.008
Boiled extract (- substrate)	0.028 ± 0.015	0.036 ± 0.010
No substrate control	0.042 ± 0.009	0.031 ± 0.015
No enzyme control	0.013 ± 0.006	0.021 ± 0.006
R.B.	0.007 ± 0.002	0.008 ± 0.002

Extracts were prepared by homogenising 25g mature mesocarp with 50 mls 0.2M Tris-HCl (pH 8.9) in a Waring Blendor. The reason for the increased molarity and pH of the buffer is given later. Experimental details are otherwise as described in Section 2.7.3.

TABLE 3.9

The Hydrolysis of HCN by High and Low m.w. Fractions of Mature Loquat Mesocarp

The high m.w. fraction (P.F. ≡ protein fraction) was collected as before. The column was then eluted with 2 x 12.5mls 0.056M Tris-HCl (pH 8.0). Both low m.w. fractions (F_1 and F_2 ≡ 1st and 2nd low m.w. fractions collected respectively) were lyophilized overnight and the residues resuspended to 3.5mls with D.D.W.

All reaction mixtures were incubated for 2 hrs at 25°C before ammonia and formamide analyses. A fresh P.F. was prepared on the 2nd day to add to the F_1 and F_2 fractions. The crude extract from day 1 and day 2 gave exactly similar results for formamide and ammonia analyses after incubation with HCN. Similar results were also found for the PFs of day 1 and 2.

Assay	Crude Extract	P.F.	F_1	F_2	0.1M NaCN buffered to pH 8.0	0.2M Tris HCl (pH 8.0)	0.05M Tris HCl (pH 8.0)	ANALYSES	
								Formamide A540nm	Ammonia A490nm
Experimental (crude extract)	0.25mls				0.5mls	0.25mls		0.481 ± 0.026	0.304 ± 0.029
No substrate control (crude extract)	0.25mls					0.25mls	0.5mls	0.428 ± 0.018	0.047 ± 0.011
Experimental (P.F.)		0.25mls			0.5mls	0.25mls		0.081 ± 0.006	0.054 ± 0.008
No substrate control (P.F.)		0.25mls				0.25mls	0.5mls	0.083 ± 0.007	0.022 ± 0.004
Experimental (F_1)			0.25mls		0.5mls	0.25mls		0.252 ± 0.046	0.190 ± 0.041
No substrate control (F_1)			0.25mls			0.25mls	0.5mls	0.258 ± 0.011	0.029 ± 0.008
Experimental (F_2)				0.25mls	0.5mls	0.25mls		0.044 ± 0.045	0.027 ± 0.000
No substrate control (F_2)				0.25mls		0.25mls	0.5mls	0.077 ± 0.018	0.017 ± 0.001
Experimental (P.F + F_1)		0.25mls	0.25mls		0.5mls			0.249 ± 0.011	0.203 ± 0.004
No substrate control (P.F. + F_1)		0.25mls	0.25mls				0.5mls	0.274 ± 0.009	0.041 ± 0.015
Experimental (P.F. + F_2)		0.25mls		0.25mls	0.5mls			0.087 ± 0.003	0.059 ± 0.011
No substrate control (P.F. + F_2)		0.25mls		0.25mls			0.5mls	0.091 ± 0.007	0.030 ± 0.009
R.B.								0.093 ± 0.006	0.020 ± 0.000

combination of high and low m.w. fractions. The composition of reaction mixtures and the results for formamide and ammonia analyses are given in Table 3.9.

No formamide production from HCN was detected for any fraction or combination of fractions. For the ammonia analyses, although a very slight increase in O.D. for the experimental P.F., above that of the control was noted, the greatest production of ammonia from HCN was observed for the experimental F_1 fraction. The inclusion of the P.F. with F_1 did not stimulate ammonia formation by F_1.

Therefore the fact that this reaction was neither:

(i) associated with the protein fraction

(ii) was not destroyed by boiling

indicates strongly that it is not enzymatic and cannot therefore be attributed to FHL activity.

3.5 DISCUSSION

Every plant investigated in this study, whether cyanogenic or not, was capable of metabolising HCN by one or more pathways; the pathway common to all plants tested was that involving β-cyanoalanine synthase. The results of the study indicated that rhodanese occurs far less commonly in plants. Rhodanese was only detected in the cyanogenic plants *S.bicolor* (Linn.) moench, *E. californica* and possibly *V. sativa* and in the non-cyanogenic plants of the genus *Brassica*. Rhodanese had previously been reported in mature leaves of *B. oleraceae* var. capitata (Tomati *et al.*, 1972) and also in *Sorghum* sp. (Myers and Fry, 1978). These findings were confirmed in the present study.

The analysis for H_2S production however, was more sensitive than that for thiocyanate production. For the β-cyanoalanine synthase assay an absorb-

ance of 0.1 corresponded to approximately 25nmoles H_2S/standard reaction mixture whereas for the rhodanese assay an absorbance of 0.1 corresponded to approximately 170nmoles thiocyanate/standard reaction mixture; in addition relatively less extract was used in the rhodanese assay.

Formamide production from HCN was not observed in any of the higher plants tested. FHL activity was, however, easily detected in extracts of *G. sorghi* when assayed under the same conditions as those used for higher plants. Although some plant extracts converted HCN to ammonia (and presumably formic acid) this reaction appeared to be non-enzymatic since the catalytic property was heat stable and was not associated with the protein fraction after G-25 chromatography.

A general trend was apparent between cyanide metabolising activity and HCN potential in higher plants; the higher the HCN potential, in general, the higher the cyanide metabolising activity. Hydrogen cyanide is a well known inhibitor of the terminal step in respiration. Since HCN is produced by the degradation of cyanogenic glycosides it may be advantageous for such plants to be capable of metabolising cyanide and for those plants which contain high levels of cyanogenic glycosides to have high levels of cyanide metabolising activity. The utilization of β-cyanoalanine synthase for the metabolism of HCN may be particularly advantageous since many plants can further metabolise its product, β-cyanoalanine, to asparagine which can then enter the general metabolism of the plant.

In a recent study, Shirai (1978) observed the conversion of HCN to ammonia in extracts of Japanese apricot (*Prunus mume*, Sieb et Zucc.) and loquat (*E. japonica*, Lindl) mesocarp. Shirais' calculations were based on the difference between experimental samples and no substrate controls. However, in a separate experiment it was shown that extracts

of loquat mesocarp consumed HCN faster than boiled extract controls, although the difference was small (approximately 1.6nmoles/mg protein/minute) and may have been due to other enzymes for which HCN is a substrate. Based on studies involving a partially purified protein fraction from loquat and work with {^{14}C}HCN, Shirai proposed that HCN is first converted to formamide by FHL and then to formic acid and ammonia, possibly via formaldoxime as an intermediate.

Using loquat mesocarp tissue of different stages of ripeness, the enzymic conversion of HCN to formamide or ammonia could not be confirmed here. However, the {^{14}C}HCN experiments of Shirai were presumably more sensitive than the colorimetric analyses of formamide and ammonia and a very low level of FHL activity may have passed undetected.

However, since the enzymic conversion of HCN to either formamide or ammonia was not observed in any of the other 14 species of higher plants examined in this study, the significance of FHL in the metabolism of HCN by higher plants could not be established.

CHAPTER 4

THE ISOLATION AND GROWTH CHARACTERISTICS OF A BACTERIUM CAPABLE OF METABOLISING ACETONITRILE

4.1. ISOLATION AND IDENTIFICATION OF THE BACTERIUM.

4.1.1. Initial Attempts at Obtaining a Bacterium Capable of Utilising Acetonitrile as the Sole C and/or N Source for Growth.

Many strains of the genus *Pseudomonas* possess an aliphatic amidase and are capable of growing on acetamide as the sole source of C and N (Clarke, 1972). Two of these *Pseudomonas* strains, *P.aeruginosa* PAC 1 (8602) and *P.putida* PPE 1 (A87) were tested for their capacity to utilise acetonitrile. It could be especially convenient if either of these organisms were able to synthesise a nitrilase enzyme because amidase negative mutants of both species exist (*P.aeriginosa* PAC 307 and *P.putida* PPN 1 (A90)*. Thus it may be possible to assay nitrilase activity in the absence of the amide hydrolysing enzyme by using the mutant strains. However, when the two amidase positive strains were subcultured into BSM containing 0.1% (v/v) acetonitrile (Section 2.2.2.(i)) there was no growth although both strains grew rapidly in the same BSM containing acetamide as sole C and N source.

Another *Pseudomonas* sp. (Group III, N.C.I.B. 10477) had previously been isolated to grow on acetonitrile as the sole source of C (Firmin & Gray, 1976). This bacterium had been kept as a lyophilised powder but after resusitation on N.A. it was subsequently found to have lost its capacity to grow on this nitrile. It was therefore necessary to isolate a bacterium, possessing nitrilase activity, from the environment by the enrichment culture technique.

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* *P.aeruginosa* PAC 1, *P.putida* PPE 1 and the amidase negative mutants were kindly provided by Professor P.H. Clarke, Department of Biochemistry, University College London.

4.1.2. Isolation of a Bacterium able to grow on Acetonitrile as the Sole Source of C and N.

BSM containing 0.1% (v/v) acetonitrile as the sole source of C and N was used throughout the procedure and sterile techniques were always employed.

Soil (0.5g) from Westfield College grounds was added to 10mls of the culture medium in a 100ml conical flask. The flask was shaken at 25°C for 6 days after which time a loopful of the resulting culture was streaked onto solid medium. After 4 days incubation at 25°C a single, discreet, colony was removed and inoculated into 10mls of liquid medium and grown for 4 days. The cycle of streaking onto solid medium, selection of a discreet colony followed by growth in liquid medium was repeated two more times.

4.1.3. Identification, Characteristics and Taxonomy of the Acetonitrile Utilising Bacterium.

The bacterium was gram positive and in view of its morphological, physiological and biochemical properties the N.C.I.B. confirmed its purity and identified it as a coryneform bacterium belonging to the genus *Rhodococcus*.

The genus *Rhodococcus* was proposed in 1977 (Goodfellow & Alderson) to include actinomycetes which had previously been classified as *Gordona*, *'Mycobacterium' rhodochrous* and the *'rhodochrous'* complex. Goodfellow & Minnikin (1977) defined members of the *Rhodococcus* genus as 'aerobic, non-sporing actinomycetes that are pleomorphic but often form a primary mycelium that soon fragments into rod and coccoid elements. A secondary mycelium is not produced but strains contain mycolic acids and have a wall chemotype IV, and the G + C content of their DNA ranges from 59-69%.'

4.2. A SURVEY OF THE ABILITY OF OTHER *RHODOCOCCUS* STRAINS TO UTILISE ACETONITRILE FOR GROWTH.

Strains from the genus *Rhodococcus*, other than the isolated acetonitrile degrader, were tested for their ability to utilise acetonitrile as the sole source of C and/or N. Acetamide was also tested as a growth substrate but only as the sole source of C and N. Ten species of *Rhodococcus* are currently recognised (Goodfellow & Schaal, 1979). The type strain* for each of these species was subcultured from N.A. slopes into 3 different media of the following composition:

Medium 1 (M1), BSM plus acetonitrile (0.2% v/v) as the sole source of C and N.

Medium 2 (M2), BSM plus acetonitrile (0.2% v/v) as the sole source of N plus glucose (0.5% w/v).

Medium 3 (M3), BSM plus acetamide (0.2% w/v) as the sole source of C and N.

Cultures (10ml) were shaken in 100ml conical flasks at approximately 20°C. Growth was determined visually after 3, 7, 14 and 21 days. Growth was usually associated with extensive flocculation so it was not practical to quantify the results by A640 measurements.

Table 4.1 shows that only one strain, namely *R.erythropolis* N11, could definately utilise acetonitrile for growth as the sole source of C and N. Only after 21 days did a second organism, *R.bronchialis* N654, show growth in m.m. containing acetonitrile as the sole source of N. Acetamide, however, proved to be a better growth substrate; 7 out of the 10 strains tested could utilise this compound as the sole source of C and N.

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*The ten type strains of *Rhodococcus* were kindly provided by Dr. M. Goodfellow, Department of Microbiology (Medical School), The University, Newcastle upon Tyne .

TABLE 4.1

Growth of the Ten Type Strains of *Rhodococcus* in Minimal Medium Containing Acetonitrile or Acetamide

Species	Strain number	Growth											
		3 days			7 days			14 days			21 days		
		M1	M2	M3	M1	M2	M3	M1	M2	M3	M1	M2	M3
R. rhodochrous	N54	-	-	-	-	-	-	-	-	-	-	-	-
R. bronchialis	N654	-	-	+	-	-	++	-	-	++	-	+	++
R. coralliinus	N657	-	-	+	-	-	++	-	-	++	-	-	++
R. coprophilus	N744	-	-	-	-	-	-	-	-	-	-	-	-
R. equi	C7	-	-	+	-	-	++	-	-	++	-	-	++
R. erythropolis	N11	-	+	++	+	++	++	+	++	++	+	++	++
R. rhodnii	N445	-	-	-	-	-	++	-	-	++	-	-	++
R. ruber	N361	-	-	+	-	-	++	-	-	++	-	-	++
R. rubroperinctus	N4	-	-	-	-	-	-	-	-	-	-	-	-
R. terrae	N659	-	-	-	-	-	-	-	-	+	-	-	++

Key: + growth
++ good growth
- no obvious growth

M1 = medium + acetonitrile
M2 = medium + acetonitrile & glucose
M3 = medium + acetamide

Tsukamura (1978), observed that 55% of all the *Rhodococcus* strains he tested could utilise acetamide in this way and concluded this was a useful character for distinguishing different *Rhodococcus* species and also *Norcadia* species.

Since the preliminary observations reported here show that the ability to grow on acetonitrile is much less common than the ability to grow on acetamide then acetonitrile utilisation may very well be useful as a highly selective character in the classification of *Rhodococcus* strains.

From these results, however, the *Rhodococcus* sp. isolate does not necessarily have to be a member of either *R. erythropolis* or *R. bronchialis* since the genus as a whole contains over 150 strains (Goodfellow & Alderson 1977) of which only the ten type strains were screened in the present investigation.

4.3. GROWTH OF THE *RHODOCOCCUS* SP. IN MINIMAL MEDIUM CONTAINING VARYING CONCENTRATIONS OF ACETONITRILE.

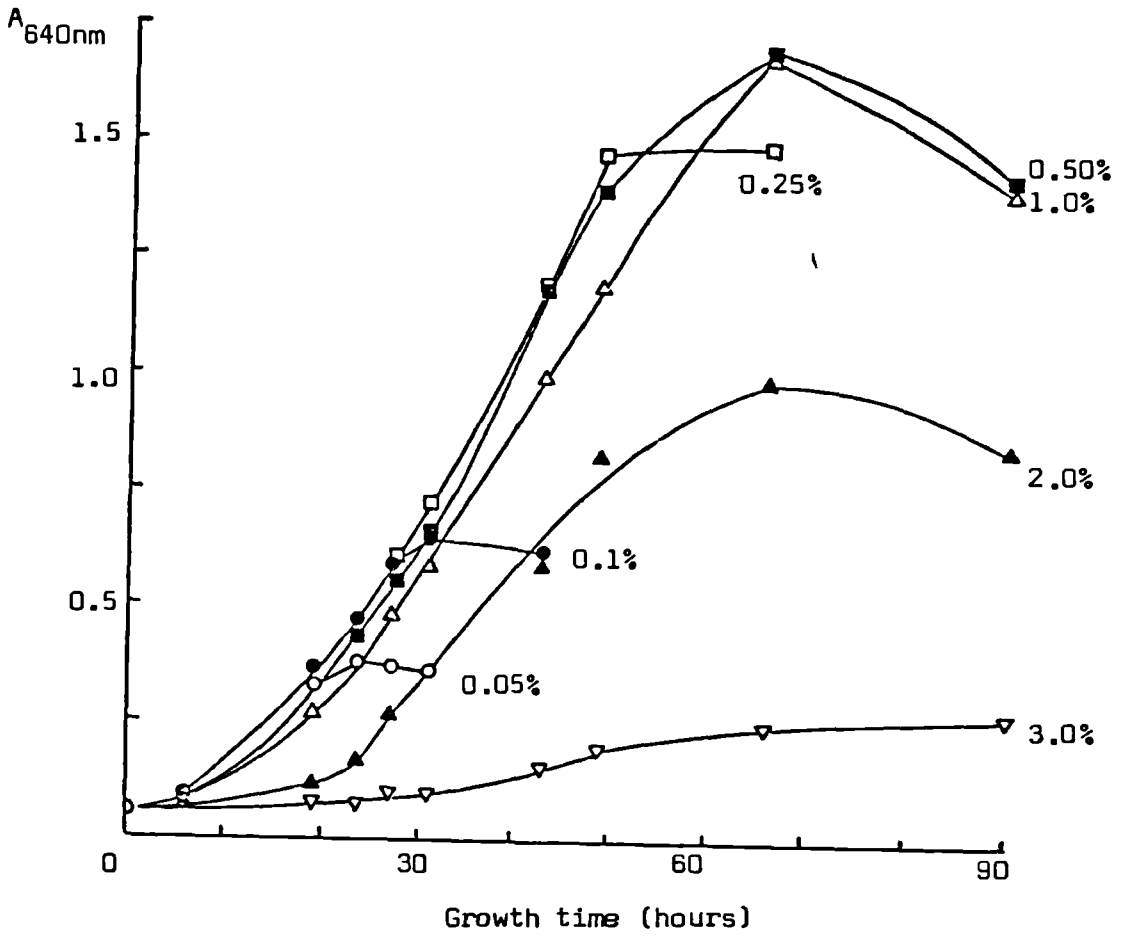
In order to determine the optimum concentration of acetonitrile for future experiments and to determine the level at which it becomes toxic the growth of the bacterium was followed in m.m. containing acetonitrile varying in concentration from 0-3% (v/v).

The results (Fig.4.1) show that the growth yield was approximately proportional to acetonitrile concentration between 0.05 and 0.25% (v/v). When acetonitrile was present at 0.5% (v/v) there was a marked reduction in molar yield while at 3% (v/v) growth was virtually inhibited.

4.4. THE UTILISATION OF VARIOUS NITRILES AND AMIDES BY THE *RHODOCOCCUS* SP. FOR GROWTH.

To investigate the utilisation of the more complex nitriles and amides a number of these compounds were screened for their ability to serve as the

FIGURE 4.1. Effect of Acetonitrile Concentration on the Growth of the *Rhodococcus* sp. in minimal Medium.



The medium used for starter cultures contained BSM plus acetonitrile (0.1% v/v). These cultures (50mls) were inoculated and grown for 2 days and then centrifuged at 12,100 x g for 10 minutes. Cells were resuspended in phosphate buffer, 0.1M, (pH 7.0) to 50mls and the test medium (95mls) inoculated with 5mls of cell suspension. The test medium was composed of BSM plus acetonitrile (0-3% v/v) as sole C and N source. Cultures were shaken and samples removed at intervals to record the A_{640}nm. Aseptic techniques were maintained throughout.

sole source of C and/or N. The test compound was always added to the medium to give a final concentration of 0.5% w/v or v/v except for urea which was present at 0.1% (w/v). The aromatic nitriles saturated the medium at this concentration and partially separated out as a second phase. The utilisation of the nitrile or amide in question was first investigated in m.m. in which the compound was present as the sole source of C and N. If growth did not occur the compound was re-tested as a N-source in m.m. containing sodium acetate or sodium succinate (1% w/v) and also as a C-source in m.m. containing $(NH_4)_2SO_4$ (0.1% w/v).

In general, nitriles and amides in the form of 10% or less v/v or w/v in BSM were sterilised by membrane filtration. However, benzonitrile and phenylacetonitrile are only sparingly soluble in aqueous solution and acrylonitrile and malononitrile, even in dilute solution, either dissolve or react with the cellulose acetate membranes. Thus, the three liquid nitriles, acrylonitrile, benzonitrile and phenylacetonitrile were added to the BSM directly with sterile pipettes while the solid malononitrile was simply added with a sterile spatula. Since none of these four compounds supported growth under any conditions the results cannot have been influenced by micro-organisms possibly present in the original nitriles. Finally, benzamide and α-phenylacetamide were also only sparingly soluble in aqueous solution. Thus, m.m. containing either of these compounds was sterilised by autoclaving the complete medium.

The results for these growth experiments are given in Tables 4.2 , 4.3 and 4.4 and summarised in Table 4.5. Tables 4.6 and 4.7 show more clearly the types of compounds utilised.

Table 4.5 shows that of the ten amides tested only one, acrylamide, failed to support growth of the *Rhodococcus* sp. when supplied as the sole source of C or N. The corresponding nitrile, acrylonitrile, was also unable to support growth. The utilisation of malonamide as the sole N-source is interesting since, assuming the pathway for degradation

TABLE 4.2

Nitriles and Amides which could be Utilised by the *Rhodococcus* sp. as the Sole Source of C & N for Growth

Medium composition	Growth time in days	A_{640}					
		0.5% (v/v) acetonitrile		0.5% (w/v) acetamide		0.5% (v/v) propionitrile	0.5% (w/v) propionamide
BSM + Test cpd. only	0	0.155	0.158	0.152	0.155	0.163 0.155	0.162 0.158
	1	0.337	0.335	1.095	1.080	0.196 0.190	0.466 0.452
	3	1.590	1.515	2.040	2.125	1.240 1.250	1.440 1.445
	7	1.225	1.210	1.630	1.675	2.325 2.300	2.130 2.175

Medium composition	Growth time in days	A_{640}				
		0.5% (v/v) butyronitrile		0.5% (w/v) n-butyramide		0.5% (w/v) α-phenylacetamide
BSM + Test cpd.only	0	0.152	0.154	0.156	0.155	0.190
	1	0.185	0.170	0.969	1.023	0.223
	3	1.195	1.410	2.285	2.175	0.910
	7	2.275	2.175	3.050	2.925	2.750

The medium used for starter cultures contained BSM plus sodium acetate (1% w/v) and $(NH_4)_2SO_4$ (0.1% w/v) . The starter culture (50mls) was grown for 4 days and then centrifuged. The cells were resuspended to 50mls with buffer, 0.1M sodium phosphate (pH 7.0). The test medium (95mls) was inoculated with 5mls of cell suspension and samples removed after 0,1,3 and 7 days and the A_{640} recorded. In most cases the experiment was done in duplicate. Sterile techniques were always employed.

TABLE 4.3

Medium composition	Growth time in days	A_{640}					
		0.5% (v/v) formamide	0.5% (w/v) malonamide	0.5% (w/v) benzamide	0.5% (w/v) nicotinamide	0.1% (w/v) urea	0.5% (w/v) 3-aminopropionitrile as fumarate salt
BSM + test cpd. only	0	0.137	0.164	0.190	0.153, 0.152	N.T	0.117, 0.117
	1	0.125	0.176	0.195	0.152, 0.153		0.925, 0.785
	3	0.125	0.200	0.195	0.143, 0.146		1.890, 1.510
	7	0.125	0.189	0.193	0.137, 0.146		2.290, 1.690
BSM + test cpd. + sodium acetate (1% w/v)	0	0.155	0.112, 0.112	0.218	0.111, 0.111	0.123, 0.123	0.113, 0.113
	1	0.377	0.560, 0.560	0.380	0.249, 0.210	1.485, 1.400	0.136, 0.147
	3	0.945	3.270, 3.280	0.750	2.830, 2.540	1.260, 1.370	1.315, 1.830
	7	1.880	2.880, 2.830	1.795	2.730, 2.560	1.970, 1.490	1.725, 1.300
BSM + test cpd. + sodium succinate (1% w/v)	0	0.106, 0.106	0.098, 0.098		0.108. 0.108		0.099, 0.099
	1	1.380, 1.225	0.835, 0.835		0.435, 0.415		0.650, 0.810
	3	- -	- -	N.T	- -	N.T	- -
	7	1.375, 1.270	1.815, 1.700		1.475, 1.215		2.195, 2.475
BSM + test cpd. + $(NH_4)_2SO_4$ (0.1% w/v)	0	0.125	0.159	0.245	0.118	N.T	N.T
	1	0.126	0.174	0.196	0.120		
	3	0.130	0.207	0.196	0.123		
	7	0.117	0.201	0.192	0.106		

3-Aminopropionitrile was added to the BSM in the form of the fumarate salt. Starter cultures which were used to inoculate media containing succinate as a C-source were grown in BSM plus sodium succinate (1% w/v) and $(NH_4)_2SO_4$ (0.1% w/v). All other experimental details are the same as those described for Table 4.2.

TABLE 4.4.

Nitriles and Amides which could not be utilised by the *Rhodococcus* sp.

as either the Sole Source of C or N for Growth

Medium composition	Growth time in days	A_{640}						
		0.5% (w/v) acrylamide	0.5% (v/v) acrylonitrile	0.5% (w/v) malononitrile	0.5% (v/v) benzonitrile	0.5% (v/v) phenyl-acetonitrile	0.5% (w/v) amino-acetonitrile
BSM + test cpd. only	0	0.158 0.173	0.163 0.154	0.185 0.177	0.235 0.240	0.230 0.217	0.189 0.189
	1	0.145 0.148	0.128 0.123	0.164 0.159	0.212 0.197	0.238 0.190	0.194 0.184
	3	0.149 0.155	0.150 0.152	0.188 0.169	0.180 0.186	0.200 0.185	0.188 0.186
	7	0.078 0.082	0.073 0.073	0.212 0.196	0.155 0.157	0.225 0.238	0.245 0.220
BSM + test cpd. + sodium acetate (1% w/v)	0	0.146 0.152	0.163 0.145	0.169 0.169	0.230 0.220	0.255	0.191 0.191
	1	0.140 0.135	0.143 0.156	0.157 0.155	0.205 0.198	0.252	0.184 0.183
	3	0.164 0.158	0.172 0.172	0.175 0.181	0.205 0.195	0.250	0.194 0.181
	7	0.076 0.089	0.084 0.093	0.202 0.188	0.147 0.149	0.320	0.224 0.211
BSM + test cpd. + $(NH_4)_2SO_4$ (0.1% w/v)	0	0.158 0.156	0.156 0.162	0.177 0.177	0.225 0.245	0.220 0.240	0.187 0.187
	1	0.125 0.138	0.126 0.135	0.149 0.146	0.218 0.215	0.201 0.201	0.183 0.183
	3	0.150 0.157	0.143 0.145	0.168 0.163	0.198 0.198	0.220 0.210	0.189 0.182
	7	0.072 0.085	0.070 0.077	0.195 0.192	0.161 0.160	0.293 0.315	0.263 0.245

Aminoacetonitrile was added to the BSM in the form of the bisulphate salt and subsequently neutralized. After 7 days incubation, media containing aminoacetonitrile had turned yellow/brown which gave rise to a small increase in absorbance, however, no increase in opalescence could be detected visually. For experimental details see Table 4.2.

TABLE 4.5

Summary of the Growth of the *Rhodococcus* sp. on various Nitriles and Amides.

Cpds. which support growth when supplied as the sole C & N source	Cpds. which support growth when supplied as the sole N source	Cpds. which could not support growth when supplied as the sole C or N source
Acetamide	Formamide (+ acetate or succinate)	Acrylamide
Propionamide	Malonamide "	
n-Butyramide	Nicotinamide "	Acrylonitrile
α-Phenylacetamide	Benzamide (+ acetate)	Malononitrile
	Urea "	Benzonitrile
Acetonitrile		Phenylacetonitrile
Propionitrile	3-aminopropionitrile(fumarate)	Aminoacetonitrile
n-Butyronitrile		

TABLE 4.6

The Utilisation of Different Types of Amides by the *Rhodococcus* sp.

Amides capable of supporting growth as sole C or N source.	Aliphatic monoamides - - - $R\text{-}CONH_2$ where R = $H, CH_3, C_2H_5, C_3H_7, NH_2$
	Aliphatic diamides - - - - - $H_2NOC\text{-}(CH_2)_n\text{-}CONH_2$ where n = 1
	Aromatic amides - - - - - - $Ar\text{-}CONH_2$ where Ar = Ø, $ØCH_2$
	Heterocyclic amides - - - $Hr\text{-}CONH_2$ where Hr =
Amides not capable of supporting growth as either sole C or N source.	Aliphatic monoamides containing a double, (vinyl), bond. - - - R = $CHCONH_2$ where R = CH_2

TABLE 4.7

The Utilisation of Different Types of Nitriles by the *Rhodococcus* sp.

Nitriles capable of supporting growth as sole C or N source	Aliphatic mononitriles- - - R-CN	where R = CH_3, C_2H_5, C_3H_7
	β-Aminonitriles - - - - - - - $H_2N-(CH_2)_n-CN$	where n = 2
Nitriles not capable of supporting growth as either sole C or N source.	Aliphatic mononitriles- - - R = CHCN containing a double, (vinyl), bond	where R = CH_2
	Aliphatic dinitriles- - - - $NC-(CH_2)_n-CN$	where n = 1
	α-Aminonitriles- - - - - - - $H_2N-\underset{\displaystyle R}{\underset{|}{C}}H-CN$	where R = H
	Aromatic nitriles - - - - - - Ar - CN	where Ar = Ø, $ØCH_2-$

involves hydrolysis, malonic acid would be formed, a well-known inhibitor of succinate dehydrogenase. Since the bacterium possesses a urease enzyme it is not surprising that it can utilise urea as the sole source of N. The involvement of the aliphatic amidase enzyme in the hydrolysis of this amide, however, is unlikely since urea is an inhibitor of this enzyme in the *Rhodococcus* sp. (Section 5.1.1) as it is for the corresponding enzyme in *P. aeruginosa*, (Kelly and Clarke, 1962).

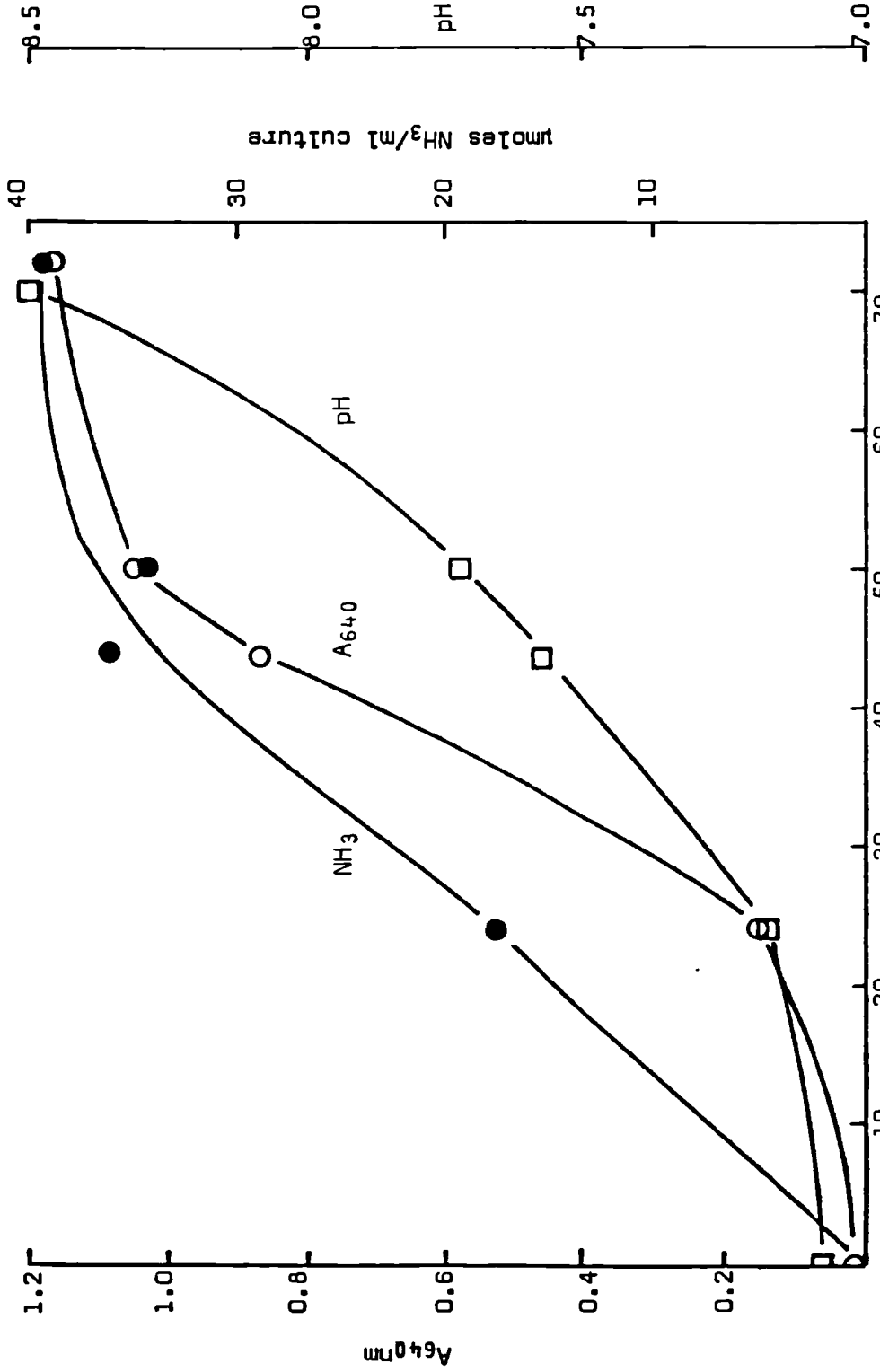
The bacterium could not, however, grow with every nitrile whose corresponding amide was utilised, e.g. benzonitrile, phenylacetonitrile and malononitrile. Aminoacetonitrile, which would be hydrolysed to glycine and ammonia,was ineffective as a growth substrate. The other aminonitrile, 3-aminopropionitrile, could support growth as a N-source indicating that it was hydrolysed to β-alanine and ammonia. 3-Aminopropionitrile may also be able to act as a C-source but this could not easily be tested since the compound was supplied (Aldrich Chemical Company) in the form of the fumarate salt.

4.5. GROWTH OF THE *RHODOCOCCUS* SP. IN MEDIUM CONTAINING ACETONITRILE AND THE ASSOCIATED CHANGES IN pH AND AMMONIA CONCENTRATION OF THE CULTURE.

Strains of *P. aeruginosa* have been shown to produce an alkaline reaction in glucose plus acetamide medium (Buhlman *et al.*, 1961) presumably due to the release of ammonia as a result of the hydrolysis of the amide. If a similar observation could be made for cultures of the *Rhodococcus* sp. growing in acetonitrile medium, this would suggest that the nitrile was hydrolysed to ammonia and so help to confirm the degradation pathway.

Cultures of the *Rhodococcus* sp. were grown in BSM plus acetonitrile (0.25% v/v) as the sole source of C and N. At intervals during growth, samples of the cultures were removed and the A_{640} and pH recorded. In addition, the concentration of ammonia in the culture supernatant was

FIGURE 4.2. The Change in pH and Ammonia Concentration of the Culture Medium During Growth of the *Rhodococcus* sp. in BSM plus 0.25% (v/v) Acetonitrile.



The medium used for the starter culture (10mls) contained acetonitrile (0.25 v/v). After 2 days growth 1ml of the starter culture was transferred to 99mls of similar medium in a 250ml conical flask. Ammonia concentration was determined by direct nesslerisation as described Section 2.7.4. Results are the average of experiments carried out on two separate cultures.

determined after removing cells by centrifugation.

Both the pH and the ammonia content of the culture increased with growth (Fig. 4.2). During the 72 hour growth period the pH rose from 7.1 to 8.5 while the ammonia concentration increased from 0.26 µmoles/ml to 39 µmoles/ml. Since acetonitrile was initially present in the medium at 0.25% (v/v) (≡47.5 µmoles/ml), approximately 82% of the ammonia which was theoretically possible from the hydrolysis of the nitrile, had been released into the medium.

4.6. DISCUSSION.

Bacteria capable of utilising acetonitrile have been isolated from a variety of environmental sources. *C.nitrilophilus* nov. sp. C-42 was isolated from activated sludge (Mimura *et al.*, 1969) whereas *N.rhodochrous* LL100-21 was obtained from barnyard soil (DiGeronimo and Antoine, 1976). Soil was also used by Arnaud *et.al.*, (1976a) from which they isolated strains belonging to the genera *Bacillus*, *Bacteridium*, *Micrococcus* and *Brevibacterium*. *Corynebacterium pseudodiphtheriticum* (N.C.I.B. 10803) was isolated from a mixture of tapwater, stored glass D.W., laboratory dust and soil and is a non-pathogenic inhabitant of the human throat (Grant and Wilson, 1973). The *Pseudomonas* sp. (Group III, N.C.I.B. 10477) isolated by Firmin and Gray (1976) was obtained simply by exposing an acetonitrile enrichment medium to laboratory air. Thus, bacteria able to degrade acetonitrile are both common and widespread in the environment.

The isolated *Rhodococcus* sp. was moderately tolerant of high acetonitrile concentrations. It was similar to *N. rhodochrous* LL100-21 in that growth was virtually inhibited at 3% (v/v) acetonitrile (DiGeronimo, 1975). Greater tolerance to acetonitrile was shown by *Corynebacterium nitrilophilus* nov. sp. C-42 for which the nitrile had to be present at 5% (v/v) before preventing growth (Mimura *et al.*, 1969). The *Pseudomonas*

sp. isolated by Firmin and Gray (1976) however was less tolerant and incapable of growing in medium containing 1% (v/v) acetonitrile.

The capacity of bacteria to utilise compounds containing a covalently bound cyanide group is by no means limited to acetonitrile. The three simplest aliphatic nitriles, acetonitrile, propionitrile and n-butyronitrile could each act as a C and N source for *N. rhodochrous* LL100-21 (DiGeronimo and Antoine, 1976) and as sole C source for *C. nitrilophilus* nov. sp. C-42 (Mimura *et al.*, 1969). Thus, these findings are similar to those for the *Rhodococcus* sp. *Corynebacterium* sp. HR3 could also utilise these 3 nitriles as sole C or N source as well as the next two homologues in the series, valeronitrile and n-capronitrile (Fukuda *et al.*, 1971). By contrast, *Arthrobacter* sp. I-9, isolated from activated sludge could grow in a medium containing propionitrile as sole C and N source (albeit rather poorly) whereas acetonitrile and n-butyronitrile could only act as sources of N (Yamada *et al.*, 1979).

The *Rhodococcus* sp. could not grow on malononitrile. A survey of the bacterial utilisation of dinitriles was carried out by Mimura *et al.*, (1969) for *C. nitrilophilus* nov. sp. C-42, but the compounds were only tested for their ability to serve as C-sources. This organism, like the *Rhodococcus* sp., could not utilise the simplest dinitrile, malononitrile, nor could it grow on glutaronitrile or 1,5-dicyanopentane. However, both succinonitrile and adiponitrile could be utilised. Thus, of the dinitriles tested only those containing an even number of C-atoms could support growth of *C. nitrilophilus* nov. sp. C-42. *N. rhodochrous* LL100-21 could also utilise succinonitrile as a C and N source (DiGeronimo and Antoine, 1976) whereas *Arthrobacter* sp. I-9 could only use it, like glutaronitrile, as a N-source (Yamada *et al.*, 1979).

Pseudomonas sp. K-9 could also utilise glutaronitrile, this time as a sole C and N source, but not acetonitrile or any other nitrile tested

(Yamada *et al.*, 1980). The pathway for the degradation of glutaronitrile was investigated by identifying the metabolites accumulating in culture filtrates when *Pseudononas* K-9 was grown in glutaronitrile-containing medium. The compounds identified were 4-cyanobutyramide, 4-cyanobutyric acid, glutaric acid and ammonia (Yamada *et al.*, 1980), suggesting that glutaronitrile was ultimately hydrolysed to the corresponding dicarboxylic acid and ammonia.

Acrylonitrile (2-propenenitrile) could not support growth of the *Rhodococcus* sp. but acted as a N-source for *N.rhodochrous* LL100-21 (DiGeronimo and Antoine, 1976). *Arthrobacter* sp. I-9, isolated by elective culture in medium containing acrylonitrile, could utilise it as a C and N source though concentrations greater than 0.24% (v/v) inhibited growth (Yamada *et al.*, 1979). *N. rhodochrous* LL100-21 was more tolerant to acrylonitrile since it would still grow at a concentration of 1% (v/v) (DiGeronimo and Antoine, 1976). Other aliphatic nitriles containing a C=C bond which have been shown to support bacterial growth as the sole source of C and/or N are hydroacrylonitrile (*N. rhodochrous*, LL100-21), 3-butenenitrile (*N. rhodochrous*, LL100-21; *Corynebacterium pseudodiphtheriticum*), methacrylonitrile and crotononitrile (*Arthrobacter* sp. I-9) (DiGeronimo and Antoine, 1976; Grant, 1973; Yamada *et al.*, 1979).

The hydroxynitrile, lactonitrile, could support growth of *C. pseudodiphtheriticum* as the sole source of C (Grant, 1973) whereas *Arthrobacter* sp. I-9 could only utilise it as a N-source (Yamada *et al.*, 1979). Although *Corynebacterium* sp. HR3 could not utilise lactonitrile at all it could grow slightly on the aminonitrile DL-α-aminopropionitrile as the sole source of C and N and also on DL-α-aminoisovaleronitrile as the sole source of N (Fukuda *et al.*, 1971). Although the *Rhodococcus* sp. could not grow on aminoacetonitrile, 3-aminopropionitrile acted as a

N-source. It is not known whether or not the latter compound could also have served as a C-source but it is interesting to note that *C. pseudodiphtheriticum* was incapable of utilising its hydrolysis product, β-alanine (Grant, 1973).

Neither of the two aromatic nitriles tested, namely benzonitrile and phenylacetonitrile, could support growth of the *Rhodococcus* sp., a finding similar to that for *C. nitrilophilus* nov. sp. C-42 (Mimura *et al.*, (1969) and for benzonitrile in the cases of *N. rhodochrous* LL100-21 (DiGeronimo and Antoine, 1976) and *Arthrobacter* sp. I-9 (Yamada *et al.*, 1979). The bacterial utilisation of benzonitrile as a source of C and N has, however been demonstrated for *N. rhodochrous* (N.C.I.B. 11216), which was isolated from river sediment by enrichment culture specifically to degrade this compound (Harper, 1977a).

From all these growth studies it is apparent that the capacity of bacteria to utilise different nitriles varies widely. Several explanations could account for these observed differences. For example the nitrilase enzyme(s) in different bacteria may possess different substrate specificities. Alternatively, some nitriles may be more toxic to some bacteria than to others and their utilisation may often depend on their concentration in the growth medium. Certainly this is true for acetonitrile (see section 4.3) and, as discussed earlier, for acrylonitrile. Since the degradation of nitriles in some bacteria requires induction (e.g. the *Rhodococcus* sp. - Ch.6 this thesis; *N.rhodochrous* LL100-21, DiGeronimo and Antoine, 1976), the ability of different nitriles to induce a nitrilase enzyme may vary in different organisms. Finally, the utilisation of a nitrile as sole C and N source by one bacterium but only as a N-source by another suggests the inability of the latter organism to utilise the carboxylic acid produced as a result of nitrile degradation.

The utilisation of nitriles is not, however, limited to bacteria. In 1951, Lamaire and Brunel grew *Sterigmatocystis nigra* in a medium containing cyanamide. Later, in 1973, Fukuda *et al.*, reported the growth of *Torulopsis candida* (GN405) on acetonitrile, propionitrile, n-butyronitrile and two hydroxynitriles, namely DL-α-hydroxyisovaleronitrile and DL-α-hydroxyisocapronitrile when each was present as the sole source of N but not C. Recently, Kuwahara *et al.*, (1980) isolated a strain of *Fusarium solani* (Mn7030) from the soil which could utilise acetonitrile, propionitrile and n-butyronitrile as good sources of N and propionitrile also as a poor source of C, similar growth characteristics to those of *Arthrobacter* sp. I-9 (Yamada *et al.*, 1979). *F. solani* (Mn7030) could also use succinonitrile and glutaronitrile as sole N-sources.

Asano *et al.*, (1981) isolated a strain of *Fusarium merismoides* (TG-1), again from soil, for its capacity to utilise triacrylonitrile (1,3,6-hexanetricarbonitrile). The simple aliphatic mononitriles would not support growth whereas adiponitrile and glutaronitrile could act as N-sources. Two other nitriles containing a double bond were utilised,namely diacrylonitrile and 2,4-dicyano-1-butene but not, surprisingly, either acrylonitrile or methacrylonitrile. When *F. merismoides* (TG-1) was grown in medium containing glutaronitrile, 4-cyanobutyric acid was identified in the culture filtrate. Similarly, when grown in the presence of diacrylonitrile, 4-cyanopentanoic acid was identified but the dicarboxylic acids corresponding to these two dinitriles were not detected. When grown in triacrylonitrile medium, both 5,7-dicyanoheptanoic acid and 4,7-dicyanoheptanoic acid accumulated but again the ultimate hydrolysis product 1,3,6-hexanetricarboxylic acid could not be detected (Asano, 1981). Presumably in these cases, the organism could only hydrolyse one of the available cyano-groups.

Finally, benzonitrile could support the growth of a strain of

Fusarium solani isolated from the soil of a bromoxynil-treated field by enrichment culture on 0.1% (v/v) benzonitrile (Harper, 1977b).

As seen from Tables 4.5, 4.6 and 4.7 the *Rhodococcus* sp. could utilise a greater number and variety of amides compared to the nitriles. Of the ten amides tested, four served as both C and N sources. Of the remainder none were utilised just as a C source wherease five could support growth in the presence of acetate or succinate. This increase in the spectrum of utilisation when the amide has only to act as N-source is not surprising since the organism has only to be capable of converting the amide to the corresponding carboxylic acid and ammonia. It does not also need to be able to synthesise the relevant catabolic pathway enzymes for the further metabolism of the acyl moiety.

The utilisation of amides by microorganisms possessing nitrilase activity, particularly when supplied as the sole source of C and N or as the sole source of N has been little investigated. Acetamide, propionamide and butyramide could each serve as a C-source for *Rhodococcus* sp., *Corynebacterium* HR3 (Fukuda *et al.*, 1971), *C. pseudodiphtheriticum* (Grant, 1973) and *F. solani* (Mn7030) (Kuwahara *et al.*, 1980). *C. pseudodiphtheriticum* could also use α-phenylacetamide as a C source but not benzamide or nicotinamide (Grant, 1973), a finding in keeping with that for the *Rhodococcus* sp.. *F. solani* (Mn7030) could also utilise formamide and succinamide poorly but adipamide quite well as N sources but could not use malonamide or n-valeramide at all (Kuwahara *et al.*, 1980). Unlike the *Rhodococcus* sp., *N. rhodochrous* LL100-21 could utilise acrylamide as a N source (DiGeronimo and Antoine, 1976).

Several species of *Pseudomonas* possess aliphatic amidases (Clarke, 1972) but these bacteria are not known to utilise nitriles. In fact two strains, namely *P. aeruginosa* PAC 1 and *P. putida* PPE 1, were shown to lack nitrilase activity (Section 4.1.1). The number of amides which can support

growth of these bacteria is fairly limited. For example, *P. aeruginosa* can utilise acetamide and propionamide as C and N sources (Clarke, 1970) but would only give a trace of growth on formamide as a N source, whereas butyramide would not support growth at all (Clarke, 1972). Some strains of *P. putida* biotype A, *P.acidovorans* and *P. cepacia* could also grow on acetamide and of these some representatives of the first two species could also grow on butyramide (Clarke, 1972). A second amidase has been identified in some *P. putida, P. acidovorans* and *P. cepacia* strains which enables growth on phenylacetamide as a C and N source (Clarke and Richmond, 1975) similar to the *Rhodococcus* sp.

Other reports on the utilisation of amides by microorganisms for growth include that of formamide as a C and N source for *Pseudomonas* sp. SL-4 (Thatcher and Weaver, 1976) and as a C source for *Pseudomonas* AM1 (Peel and Quayle, 1961) and *Thiobacillus novellus* (Chandra and Shethna, 1977). *Aspergillus nidulans* could grow with formamide only as a N source but with acetamide as both C and N source (Hynes, 1970). *Mycobacterium smegmatis* could also utilise acetamide as a C source as well as butyramide, benzamide and nicotinamide (Draper, 1967).

Recently Gresshoff (1981) reported the growth of the unicellular green algae *Chlamydomonas reinhardi* on acetamide as the sole source of C and N. This organism could also utilise formamide, propionamide, butyramide, urea and glutamine as N-sources but not several other amides tested including acrylamide.

In 1978 Tsukamura listed characters useful for recognising members of the species *Rhodococcus sputi* sp. nov. Tsukamura. These included the utilisation of acetamide as the sole C and N source and nicotinamide, succinamide and urea as sole N sources whereas benzamide and isonicotinamide could not support growth at all. Since benzamide could be utilised by the *Rhodococcus* sp. (this thesis) it is obviously not a strain of *R.sputi*.

CHAPTER 5

ACETONITRILASE AND ACETAMIDASE ACTIVITIES OF THE *RHODOCOCCUS* SP.

5.1. DEVELOPMENT OF THE NITRILASE AND AMIDASE ASSAYS.

Having isolated a bacterium capable of utilising several nitriles and amides the next step was to develop a method for assaying the nitrilase and amidase enzymes.

5.1.1. Attempts to Measure Nitrilase and Amidase Activities Using the Hydroxamate Reaction.

The initial step in the metabolism of acetonitrile in other bacteria has been shown to be hydrolysis to the corresponding amide (Firmin & Gray, 1976; DiGeronimo & Antoine, 1976). One method of detecting acetamide is to react it with hydroxylamine to form acethydroxamate which gives a red/brown iron chelate complex with ferric chloride. Two different methods of the hydroxamate test were investigated to determine which was the most sensitive for acetamide.

The first method was that described by Snell (1961) in which the amide solution is mixed with alkaline hydroxylamine and incubated at 25^{o}C for 6 hours. The colour is developed by the addition of HCl and ferric chloride and the absorbance read immediately at 540nm. Solutions of acetamide ranging from 0-300µg/ml (approximately 0-5mM) gave a linear calibration between 0-1.08 O.D. units.

The second method was that described by Firmin & Gray (1975) in which the amide solution is mixed with hydroxylamine hydrochloride in glycerol and heated for 60 minutes at 110^{o}C. When cool the colour is developed by the addition of HCl and ferric chloride and the absorbance read immediately at 520nm. Solutions of acetamide ranging from 0-500µg/ml (approximately 0-8.5mM) gave a linear calibration between 0-0.4 O.D. units.

Thus, the first method (Snell, 1961) was 4.5 times more sensitive than the second and was therefore chosen for preliminary experiments aimed at

investigating the metabolism of acetonitrile and acetamide.

Acetamide hydrolysis.

In the first experiment the metabolism of acetamide was investigated in a cell-free extract of the *Rhodococcus* sp. (Fig.5.1). The rate of acetamide disappearance was constant for the first 30 minutes, the rate then decreased and the amide was exhausted within 120 minutes. Thus the disappearance of acetamide from reaction mixtures as recorded by hydroxamate formation was a satisfactory method for assaying the amidase.

In order to determine the activity of the nitrilase by measuring its presumed product acetamide, it would be necessary to inhibit the second enzyme in the pathway to prevent further metabolism of the amide. In 1962, Kelly & Clarke reported that urea inhibited the aliphatic amidase of *P. aeruginosa*. The effect of urea on the corresponding *Rhodococcus* enzyme was therefore investigated.

A cell-free extract was incubated with acetamide at an overall concentration of 8.5mM in the presence of 0-166mM urea. The disappearance of acetamide with time from five different reaction mixtures is illustrated in Fig.5.2. The results show that the molar ratio of urea to acetamide must be in the order of 20:1 to completely inhibit the amidase activity of the extracts.

Acetonitrile hydrolysis.

In the first attempt to assay nitrilase activity by hydroxamate formation, the *Rhodococcus* sp. was grown, harvested and resuspended as in the last two experiments but was sonicated for a total of 10 minutes. Experimental reaction mixtures which contained 2.38 mg/ml protein $\pm$ 166mM urea and 8.5mM acetonitrile were equilibrated and incubated as for Fig.5.2.

FIGURE 5.1. Time Course of Acetamide Hydrolysis by a Cell-free Extract of *Rhodococcus* sp. as Recorded by the Hydroxamate Reaction.

The bacterium was grown in a total of 1ℓ BSM plus sodium acetate (1% w/v) and acetonitrile (0.1% v/v), harvested by centrifugation and resuspended to approx. 250mls in D.W. The cell-free extract was prepared as described in Section 2.4.1; the total sonication time being 3 minutes. The experimental reaction mixture consisted of 1.57 mg/ml protein (as determined by the A_{280} with egg albumen as standard) and 500µg/ml (8.5mM) acetamide. The extract and substrate solutions were equilibrated separately at 25°C for 5 minutes before mixing. Boiled extract controls and no substrate controls were also run. All mixtures were incubated at 25°C and at intervals samples were removed and the reaction for the experimental and no substrate controls terminated by boiling for 15 minutes. Precipitated protein was removed by centrifugation and the amide concentration determined by the method of Snell (1961). The controls gave constant A_{540} readings throughout the 120 minutes incubation. Acetamide in the experimental reaction mixture was determined by comparison with standards after subtracting the value for the no substrate control.

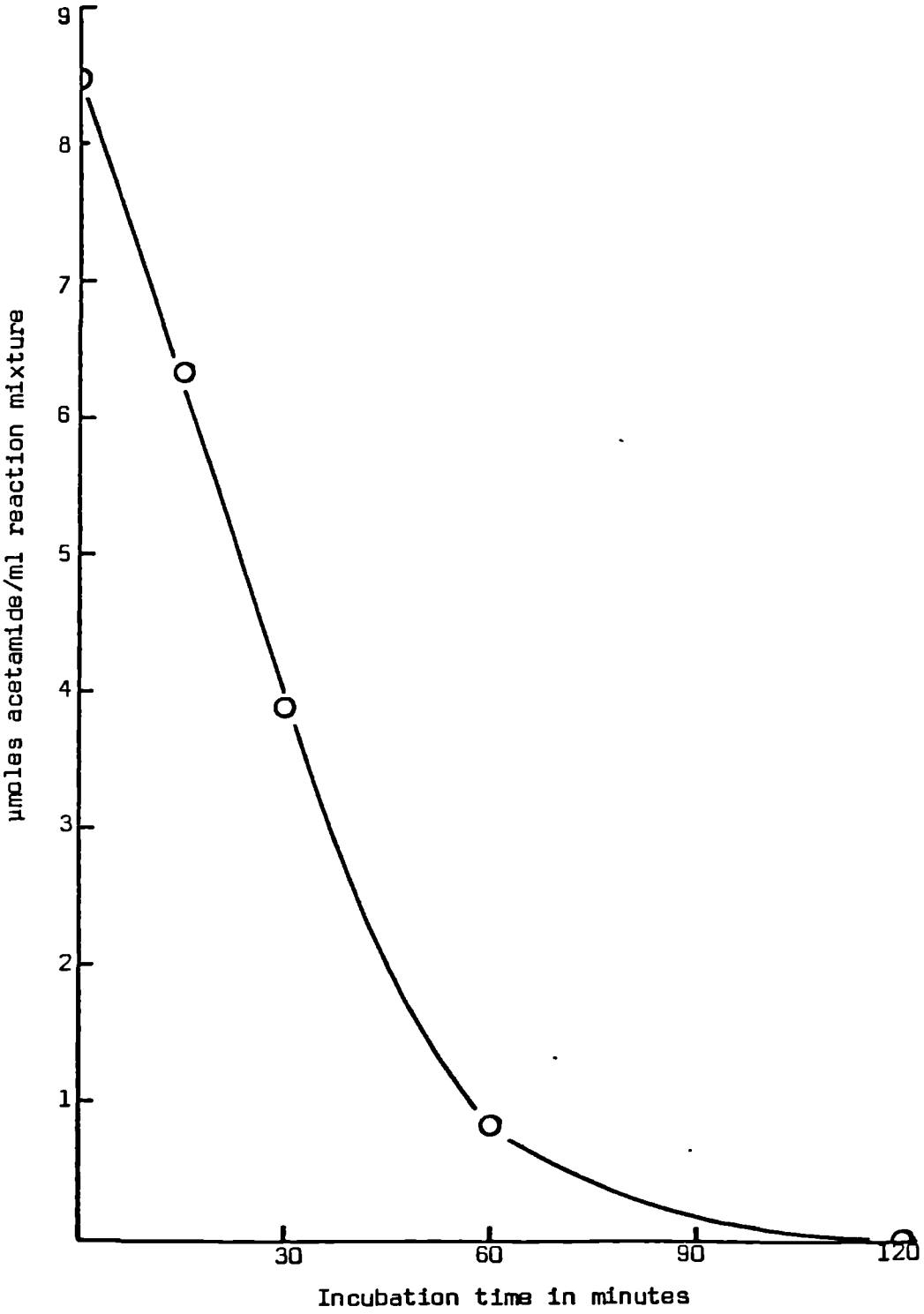
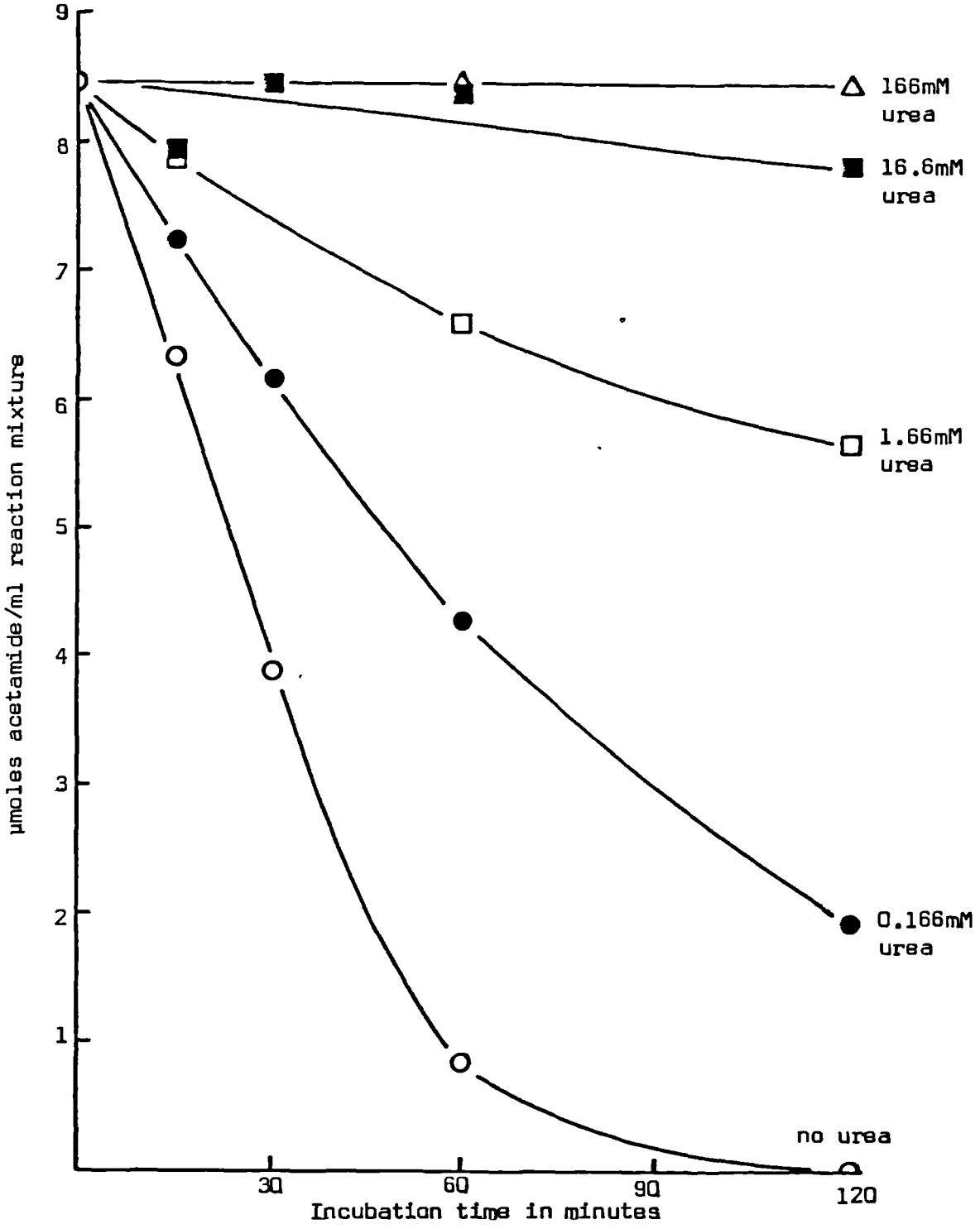

µmoles acetamide/ml reaction mixture
9
8
7
6
5
4
3
2
1
30
60
90
120
Incubation time in minutes

FIGURE 5.2. The Effect of Urea on the Hydrolysis of Acetamide by a Cell-free Extract of the *Rhodococcus* sp.



The experiment was carried out as described for Fig.5.1 except that the extract (17mls) was equilibrated for 10 minutes in the presence of urea (1ml of 0-3.32M urea) prior to the addition of the substrate (2mls of 170mM acetamide).

Samples were removed after 0, 15, 30, 60 and 120 minutes incubation and treated as before (see Fig. 5.1). There was no difference in the A_{540} values between any of the samples removed from the experimental reaction mixture during the 120 minutes incubation. Similar results were obtained for boiled extract controls.

There are several possible explanations for this negative result. Urea might inhibit nitrilase activity while, in its absence, acetamide might fail to accumulate. Alternatively, the nitrilase activity of the extract might have been too low to be detected under these conditions.

The second attempt was a duplicate of the first except that the overall substrate concentration was increased to 95mM in case the K_m for the nitrilase enzyme was exceptionally high. However, it was found that this concentration of acetonitrile gave a large positive hydroxamate reaction when analysed by the method of Snell (1961): experimental samples, boiled extract controls and no extract controls, all gave A_{540} readings higher than 1.3 O.D. units. Thus, acetonitrile either reacts directly to form the amidoxime, which gives a coloured ferric ion complex (Robertson *et al.*, 1964) or the nitrile is spontaneously hydrolysed to yield some acetamide during the 6 hour incubation period in the presence of the alkaline hydroxylamine reagent.

The latter explanation was probably correct since 95mM aqueous acetonitrile gave a negative result when subjected to the hydroxamate test of Firmin & Gray (1975) which is not carried out under alkaline conditions. Thus this second method was employed in all subsequent attempts to determine nitrilase activity by the hydroxamate reaction.

In the third attempt to measure acetonitrilase activity, the cell suspension was concentrated 15 fold prior to sonication. Reaction mixtures were similar to the last experiment but contained 11mg/ml protein and were incubated for 2 and 4 hours. Samples were removed and treated as described

for Fig. 5.1. except for the different hydroxamate method employed. There was no significant difference, however, between the A_{520} readings for the experimental samples and controls, with or without urea.

For the fourth attempt, reaction mixtures were incubated for 20 hours. Bacterial contamination during this long incubation period was prevented by the addition of streptomycin. From the results shown in Table 5.1. it appears that for the first time the formation of acetamide from acetonitrile by a cell-free extract of the *Rhodococcus* sp. was demonstrated. The A_{520} values for the experimental samples were greater than those of the corresponding controls whether incubated with or without urea although greater differences were observed in the presence of urea. Even so, after 20 hours incubation in the presence of urea, a conversion of only 1.7% of the substrate, acetonitrile, to acetamide was detected.

In the fifth experiment, the effect of the pH of the reaction mixture on the formation of acetamide from acetonitrile was investigated. Cell-free extracts were incubated for 20 hours with acetonitrile, urea and buffer at pH's ranging from 4 - 8. Positive results were obtained for all experimental reaction mixtures when compared to the controls (Table 5.2.). However, the pH of all the experimental and no substrate control samples increased during the 20 hour incubation period.

The experiment was repeated at a slightly different pH range and this time samples were removed for analysis after 2, 4, 6 and 8 hours (Table 5.3). Again, positive results were obtained for all experimental samples although at pH 10.1 the boiled control also gave high A_{520} readings. The results indicated that the concentration of amide in experimental mixtures was less after 8 hours incubation than after only 2 hours incubation. Again, the pHs of the experimental reaction mixtures increased during the incubation as did those of the no substrate controls.

These changes could be explained by the presence of urease in the

TABLE 5.1

Acetonitrile Hydrolysis by a Cell-free Extract of *Rhodococcus* sp. as Recorded by the Hyxroxamate Reaction.

Assay	± Urea	A_{520}	Concentration of acetamide in experimental sample (n moles/ml)
Experimental	-	0.311 ± 0.005	0.54 ± 0.27
No substrate control	-	0.267 ± 0.014	
Boiled control	-	0.277 ± 0.003	
Experimental	+	0.408 ± 0.004	1.66 ± 0.10
No substrate control	+	0.292 ± 0.001	
Boiled control	+	0.294 ± 0.001	

The bacterium was grown as described in Fig. 5.1, harvested, resuspended in D.W. and sonicated for a total of 10 minutes. The experimental reaction mixture consisted of 9.8 mg/ml protein ± 166mM urea and 95mM acetonitrile. Reaction mixtures were set up as described for Fig. 5.2 except for the nature of the substrate. After 20 hours incubation at 25°C, samples were treated as described in Fig. 5.1 except for the different hydroxamate method employed. Results are the average of 4 analyses for each assay. Acetamide concentration in experimental samples was determined by comparison with standards after subtracting the value for the appropriate boiled control.

TABLE 5.2.

The Effect of pH on the Formation of Acetamide from Acetonitrile by Cell-free Extracts of *Rhodococcus* sp. : Experiment A

pH of reaction mixture at start of experiment	Assay	A_{520}	pH of reaction mixture after 20 hours incubation
4	Experimental	0.262 ± 0.008	6.2
"	No substrate control	0.125 ± 0.011	6.2
"	Boiled control	0.140 ± 0.006	4.0
5	Experimental	0.657 ± 0.028	8.8
"	No substrate control	0.169 ± 0.008	8.7
"	Boiled control	0.075 ± 0.003	5.0
6	Experimental	0.615 ± 0.011	8.8
"	No substrate control	0.092 ± 0.006	8.9
"	Boiled control	0.136 ± 0.014	6.0
7	Experimental	0.670 ± 0.026	9.1
"	No substrate control	0.032 ± 0.004	9.0
"	Boiled control	0.142 ± 0.018	7.0
8	Experimental	0.647 ± 0.048	9.2
"	No substrate control	0.007 ± 0.006	9.2
"	Boiled control	0.212 ± 0.026	8.0

The bacterium was grown and harvested as detailed in Fig. 5.1. and the cell suspension concentrated 13-fold prior to sonication. The cell suspension was sonicated and cell-free extract obtained as for Fig. 5.1. The experimental reaction mixture consisted of 3.5mls cell-free extract, 0.5mls aqueous acetonitrile (1.9M), 5mls buffer (0.1M citric acid/0.2M Na_2HPO_4, varying in pH from 4-8), 0.5mls aqueous urea (3.32M) and 0.5mls streptomycin (2mg/ml). The pH of each reaction mixture was adjusted to the desired value of zero-time. After 20 hours incubation, samples were treated as for Fig. 5.1 except for the different hydroxamate method employed. Results are the average absorbance values from duplicate assays after subtracting the value for the reagent blank.

TABLE 5.3

The Effect of pH on the Formation of Acetamide from Acetonitrile by Cell-free Extracts of *Rhodococcus* sp. : Experiment B.

pH of reaction mixture at start of experiment	Assay	Incubation time in hours 2	4	6	8 (A_{520} readings)	pH of reaction mixture after 8 hrs incubation
5	Experimental	0.410	0.495	0.398	0.345	5.8
"	No substrate control	0.195	0.330	0.274	0.231	5.8
"	Boiled control	0.238	0.210	0.174	0.129	5.0
6	Experimental	0.460	0.445	0.465	0.435	8.4
"	No substrate control	0.237	0.240	0.145	0.114	7.7
7	Experiment	0.700	0.750	0.555	0.258	9.2
"	No substrate control	0.065	0.050	0.021	0.005	9.2
8	Experimental	1.035	0.500	0.365	0.250	9.3
"	No substrate control	0.006	0.000	0.000	0.000	9.3
9.2	Experimental	0.875	1.375	0.695	0.650	9.2
"	No substrate control	0.130	0.130	0.109	0.110	9.3
10.1	Experimental	1.375	0.925	0.650	0.500	9.6
"	No substrate control	0.121	0.120	0.120	0.121	9.6
"	Boiled control	0.643	0.835	0.555	0.345	10.0

Experimental details are essentially the same as those described for Table 5.2 except that the cells were resuspended in 0.05m sodium phosphate buffer (pH 7.2) prior to sonication. In addition all components in reaction mixtures were made up in buffer of the appropriate pH for that assay. Assays carried out at pH 9.2 and 10.1 were done in the presence of 0.1m $Na_2CO_3/NaHCO_3$ buffer.

extracts: ammonia, released by urea hydrolysis, could account for the observed increases in pH. Moreoever, the corresponding decrease in urea concentration may have been such as to relieve amidase inhibition and so reduce acetamide accumulation as actually observed. The situation would be further complicated when the changes in pH and/or ammonia concentration changed the absolute and relative activities of the nitrilase and amidase enzymes.

The bacterium used here did in fact contain urease: the presence of this enzyme was later confirmed by the N.C.I.B. and used as one of the criteria for classifying the isolate as a species of *Rhodococcus*.

Thus nitrilase activity could not be simply and satisfactorily measured in cell-free extracts by the hydroxamate reaction applied as described here. Attempts were therefore made to develop a more satisfactory alternative method. Nevertheless these preliminary investigations were valuable in that they confirmed that an amide was an intermediate in acetonitrile breakdown. Moreover, they strongly suggested that urea does inhibit *Rhodococcus* amidase.

5.1.2. The Measurement of Nitrilase and Amidase Activities by Ammonia Formation.

The amidase of *P.aeruginosa* has been studied by ammonia formation in both cell-free extracts and intact cell suspensions (Kelly & Clarke, 1962). With intact cells, the ammonia formed from acetamide hydrolysis was released into the medium. Theoretically, the nitrilase activity of the *Rhodococcus* sp. could also be estimated by ammonia production by making use of the natural coupling between the nitrilase and amidase enzymes.

Thus, in the first attempt to measure both the nitrilase and amidase activities of the *Rhodococcus* sp. by ammonia formation, intact cell suspensions were incubated separately with acetonitrile and acetamide. The formation of

ammonia was estimated by direct nesslerisation. The results (Fig. 5.3) showed that indeed it was possible to assay both the nitrilase and amidase activities *in situ* in this way. The rate of ammonia formation from aceto-nitrile and acetamide by the intact cell suspension of *Rhodococcus* sp. was calculated from this experiment to be 277 and 390 nmoles ammonia produced/mg dry wt. cells/minute, respectively.

Further aspects of these assays with intact cell suspension involving ammonia analyses, e.g. quantitative nature of acetamide hydrolysis and validity of the coupled assay are dealt with later in Sections 5.3.4. and 5.4.

5.2. THE RECOVERY OF THE NITRILASE AND AMIDASE ACTIVITIES IN CELL-FREE EXTRACTS OF THE *RHODOCOCCUS* SP.

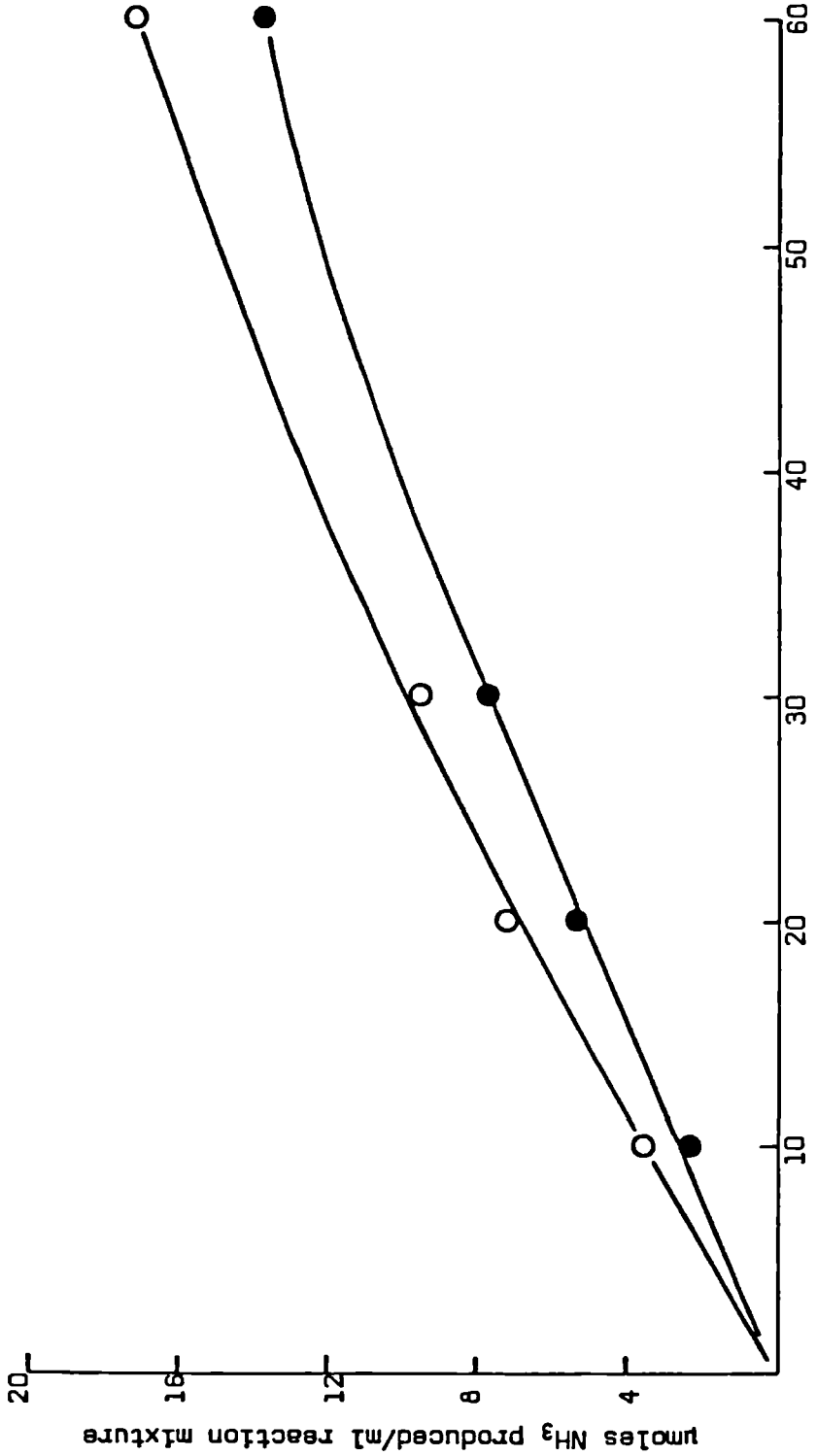
5.2.1. Cell-free Extracts Prepared by Sonication.

In the first attempt to measure nitrilase and amidase activities in a cell-free extract, by ammonia formation, a suspension of the *Rhodococcus* sp. was sonicated for a total of 5 minutes. The "soluble" and "particulate" fractions were recovered as described in Section 2.4.1. and both these fractions plus the original cell suspension were assayed for nitrilase and amidase activity as described in Section 5.1.2.

As shown in Table 5.4, very little nitrilase and amidase activity was lost during sonication. However, only 9.4% of the amidase and 6.2% of the nitrilase activity originally present in the intact cells was recovered in the "soluble" fraction. Therefore these results suggested that either the enzymes were particle-bound or else the sonication treatment was not very effective in disrupting the cells.

When the "soluble" fraction, obtained after centrifugation at 17,400g,

FIGURE 5.3. The Production of Ammonia from Acetonitrile and Acetamide by Intact Cells of the *Rhodococcus* sp.



Ammonia production was estimated with acetonitrile (■-■) or acetamide (O-O) as substrate. The bacterium was grown in 5 ℓ of BSM plus acetonitrile (0.1% v/v) and sodium acetate (0.5% w/v) and harvested and resuspended as described in Section 2.7.4. The composition of reaction mixtures is described in Section 2.7.4 - modification 1. The cell suspension was diluted 5-fold prior to both the nitrilase and amidase assays. At intervals samples were removed and the ammonia concentration determined as described in Section 2.7.4 - "direct method". Enzyme activities in experimental samples were calculated after subtracting the value for the appropriate boiled control.

TABLE 5.4

The Distribution of Nitrilase and Amidase Activities in the "Soluble" and "Particulate" Fractions After Sonication of the *Rhodococcus* sp.

Assay	Fraction	Comparative activity (μmoles NH_3produced/ ml reaction mixture/ minute)	% Activity recovered in each fraction	Total % activity recovered in the "soluble" plus "particulate" fractions.
Nitrilase	Intact cells	0.650	100	
	"Particulate"	0.583	89.7	95.9
	"Soluble"	0.040	6.2	
Amidase	Intact cells	4.49	100	
	"Particulate"	3.83	85.3	94.7
	"Soluble"	0.42	9.4	

The bacterium was grown, harvested and resuspended as described for Fig. 5.3. The cell suspension was sonicated and the fractions separated as stated in the text. Fractions were assayed for both nitrilase and amidase activity as described in Section 2.7.4 - modification 1. Samples were removed after 15, 30 and 60 minutes incubation and the concentration of ammonia determined by direct nesslerisation as described in Section 2.7.4. Ammonia formation in experimental samples containing the "soluble" fraction were calculated after subtracting the values for the appropriate boiled control whereas,for samples containing the "particulate" fraction or intact cells,calculations were made after subtracting the appropriate zero-time experimental samples. Activities were determined from the resulting progress curves. The values obtained for the boiled controls and no substrate controls for a given assay and fraction remained constant throughout the 60 minutes incubation. The different fractions were diluted by different amounts prior to assay. However, in order to make the results comparable to each other the activities given are those calculated for a reaction mixture containing 0.2ml of undiluted fraction/ml. Results are the average of duplicate assays.

in a separate experiment, was re-centrifuged at approximately 100,000g for 30 minutes, 100% of both the nitrilase and amidase activities remained in the supernatant fraction (Table 5.5). This result suggests that neither of these enzymes present in the "soluble" fraction were particulate. Thus it seemed worthwhile to attempt to solubilise a larger proportion of these enzymes.

First of all the effect of the pH at which the bacterium was sonicated was investigated. Cells were resuspended in a range of sodium phosphate buffers (0.1M) varying in pH from 5.8 - 8.0. The "soluble" fractions, obtained after sonication and centrifugation, were assayed for nitrilase and amidase activities at pH 7.0 (Table 5.6).

The A_{280} readings indicate that the pH of the extracting buffer had little effect on the total amount of cellular material released into the medium. However, nitrilase activity was greater in the extract prepared by sonication at pH 7.0. Amidase activity, however, continued to increase in extracts prepared in buffers of increasing pH. These results may reflect the stability of the enzymes in buffers of differing pH. From these results it was decided to continue preparing cell-free extracts at pH 7.0.

The effect of the molarity of the extracting buffer was studied next. Cells were resuspended in three buffers (sodium phosphate, pH 7.0) varying in molarity from 0.002 - 0.167M. Each suspension was sonicated and each "soluble" fraction assayed for nitrilase and amidase activities.

Fig. 5.4 and Table 5.7 show that increasing the molarity of the extracting buffer resulted in increased nitrilase and amidase activity. However, the A_{280} readings suggest that these increases in activity cannot be explained by a corresponding increase in cellular material released into the medium. Thus, either the high salt concentrations exert a stabilising effect on these enzymes or else selectively promote their release during sonication.

TABLE 5.5.

Nitrilase and Amidase Activities in the "Soluble" Fraction obtained After Successive Centrifugation at 17,400g and 100,000g

Assay	Centrifugation conditions	Comparative activity (μmoles NH_3 produced/ml reaction mixture/ minute)
Nitrilase	17,400g : 15 mins.	0.109 ± 0.008
	100,000g : 30 mins.	0.113 ± 0.008
Amidase	17,400g : 15 mins.	1.27 ± 0.02
	100,000g : 30 mins.	1.34 ± 0.02

Experimental details were essentially the same as those described for Table 5.4 except the amount of ammonia formed in reaction mixtures was measured after 0 and 10 minutes incubation for the amidase assay and after 0 and 30 minutes for the nitrilase assay. Activities were calculated after subtracting the value for the appropriate zero-time control. Both fractions were diluted 4-fold prior to the nitrilase assay and 8-fold prior to the amidase assay but the results are expressed in comparative terms as described for Table 5.4 Results are the average of duplicate assays.

TABLE 5.6

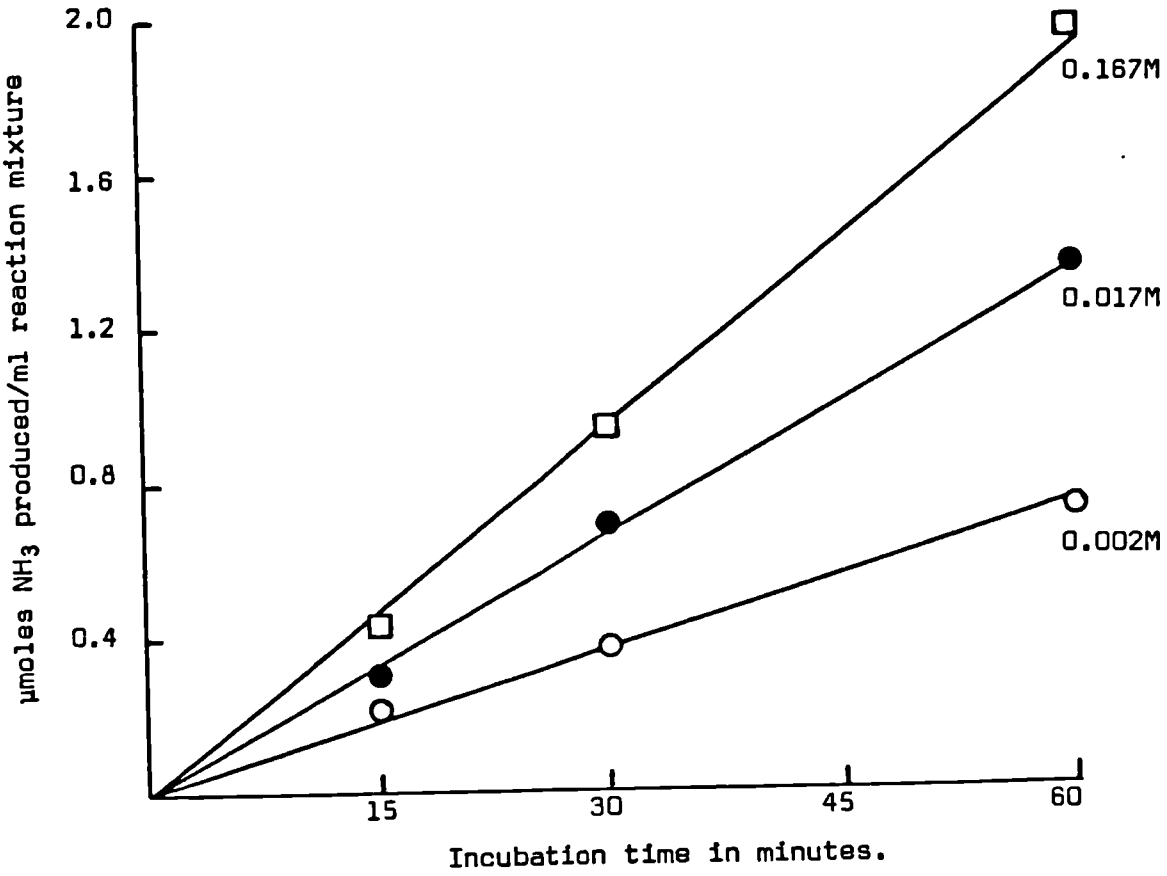
The Effect of the pH of the Extracting Buffer on the Nitrilase and Amidase Activities in the "Soluble" Fraction After Sonication.

Assay	pH of cell suspension sonicated	Activity (nmoles NH_3 produced/ml reaction mixture/ minute	A_{280} of extract
Nitrilase	5.8	11.3	4.12
	6.4	14.8	4.12
	7.0	16.2	4.22
	8.0	9.3	4.32
Amidase	5.8	12.7	4.12
	6.4	21.3	4.12
	7.0	48.0	4.22
	8.0	58.0	4.32

The bacterium was grown and harvested as described for Fig.5.3. Cells were resuspended in D.W. to 100mls. This suspension was divided equally into 4 samples and to each was added 25mls of sodium phosphate buffer (0.2M) at the stated pH. Each 50ml suspension was sonicated for a total of 5 mins. and the "soluble" fractions obtained as described in Section 2.4.1. To 2mls of each undiluted extract was added 6mls of 0.1M sodium phosphate buffer (pH 7.0) and the pH adjusted to 7.0. The substrate solution was then added (Section 2.7.4 - modification 1) and reaction mixtures incubated for 60 minutes when assaying nitrilase activity or 15 minutes when assaying amidase activity. Ammonia was determined as for Fig. 5.3 and activities calculated after subtracting the value for the appropriate boiled control.

N.B. - there was probably an 18% loss of amidase activity prior to assay since the extracts were stored at $4^{o}C$ for 1 day prior to carrying out amidase assays (see Section 5.3.2.).

FIGURE 5.4. Nitrilase Activity in "Soluble" Fractions Prepared in Buffers of Different Molarities.



The bacterium was grown and harvested as described for Fig. 5.3. The cells were resuspended to 30mls with 0.002M sodium phosphate buffer (pH 7.0) and divided into 3 equal aliquots. To each was added 50mls of buffer at a concentration of 0.002M, 0.02M or 0.2M. Each suspension was sonicated for 5 minutes and the "soluble" fractions obtained as in Section 2.4.1. Reaction mixtures were as described in Section 2.7.4 - modification 1. None of the fractions were diluted prior to assay and the ammonia concentration was determined as for Fig. 5.3. The activity in experimental samples was calculated after subtracting the value for the appropriate boiled control.

TABLE 5.7

Amidase Activity in "Soluble" Fractions Prepared by Sonication in Buffers of Different Molarities

Molarity of extracting buffer	Activity (nmoles NH_3 produced/ml reaction mixture/minute)	A_{280} of extract
0.002 M	97.3	2.46
0.017 M	100.0	2.24
0.167 M	124.7	2.12

Experimental details are essentially the same as for Fig. 5.4 except for the nature of the substrate and also the amidase activity was determined after a 15 minute incubation period only.

All these early sonication experiments were done using equipment with a maximum power output of 75W. Although this had been used in attempts to investigate the effect of sonication time on the % release of nitrilase activity into the "soluble" fraction, very little activity could be detected making the results inconclusive. Eventually a new sonicator with a maximum output of 150W became available and the experiment was repeated to give the graphs shown in Fig. 5.5.

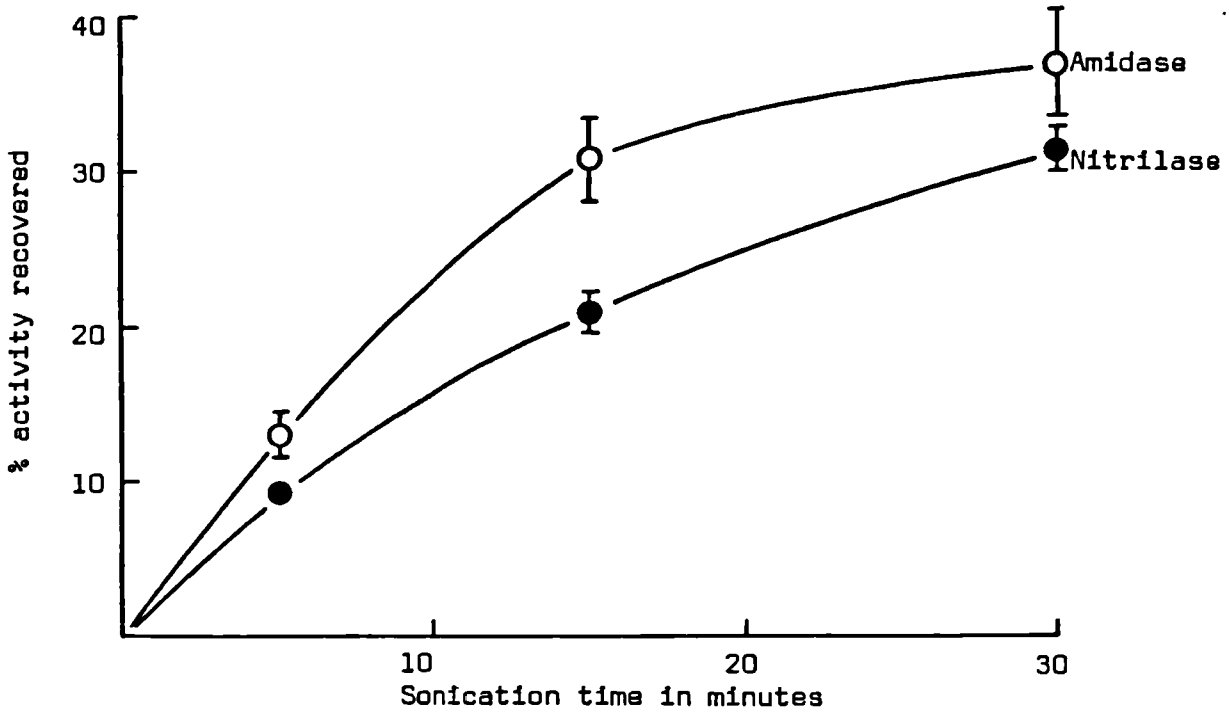
A comparison with Table 5.4 shows that for a total sonication time of 5 minutes the new equipment resulted in a significantly greater proportion of the nitrilase and amidase activities recovered in the "soluble" fraction. Moreover, the percentages of both enzymes in the "soluble" fraction increased with increasing sonication time although not proportionally so. Sonicating the cell suspension for more than 30 minutes was not considered practical due to the time it would take to prepare the extract (>1 hour) and also because such long sonication times caused excessive erosion of the probe tip. In any case Fig. 5.5 suggests that little more than 40% of either the nitrilase or amidase activities could be recovered in the "soluble" fraction however prolonged the sonication period. The non-linear relationship observed between sonication time and enzyme activities in the "soluble" fraction may have been a result of progressive denaturation of the enzymes exposed to sonication treatment, e.g. by heat and/or oxidation.

5.2.2. Cell-free Extracts Prepared Using a French Press.

In another attempt at improving the % of nitrilase and amidase activities in the "soluble" fraction, extracts were prepared by passing a cell suspension through a French Press.

Activities recovered in the "soluble" and "particulate" fractions compared to those of the original cell suspension are given in Table 5.8.

FIGURE 5.5 The Effect of Sonication Time on the Percentage of Nitrilase and Amidase Activities Recovered in the "Soluble" Fraction.



The bacterium was grown in 5 l of BSM plus 0.25% (v/v) acetonitrile and harvested as described in Section 2.7.4. Cells were resuspended to 200mls in 0.2M sodium phosphate buffer (pH 7.0). Samples (50mls) of this cell suspension were sonicated for 5, 15 & 30 minutes and the soluble fractions recovered as described in Section 2.4.1. The original cell suspension and the resulting "soluble" fractions were assayed as described in Section 2.7.4 - modification 2. Ammonia concentration was determined as in Section 2.7.4 - "direct method" and activities calculated after subtracting the appropriate zero-time control from experimental samples. No substrate controls were also carried out; the values obtained remained constant during the incubation period. Results are the average of duplicate assays. The % activities recovered in the "soluble" fractions were calculated by comparison to the intact cell suspension.

TABLE 5.8

The Distribution of Nitrilase and Amidase Activities in the "Soluble" and "Particulate" Fractions After Passing a Suspension of the *Rhodococcus* sp. through a French Press.

Assay	Fraction	Comparative activity (μmoles NH_3 produced /ml reaction mixture /minute)	% Activity recovered in each fraction	Total % activity recovered in the "soluble" plus "particulate" fractions
Nitrilase	Intact cells	1.3	100	
	"Particulate"	0.514	38.2	43.0
	"Soluble"	0.065	4.8	
Amidase	Intact cells	2.168	100	
	"Particulate"	1.214	56.0	77.8
	"Soluble"	0.472	21.8	

Experimental details were essentially the same as those described for Table 5.4 except for the method of extract preparation, assay temperature (35^{o}C) and incubation times which for this experiment was 10 minutes in the case of nitrilase assays and 5 minutes for amidase assays. Furthermore, activities were determined after subtracting the value for the appropriate no substrate control. Enzyme activities are expressed in comparative terms as described for Table 5.4.

The proportion of the amidase activity recovered in the "soluble" fraction, approximately 22%, compared favourably with that given by sonication, especially when the 75W sonicator was being used. However, greater than 20% of the total amidase activity was lost when the cell suspension was disrupted with the press. Results for the nitrilase assays showed that only a small percentage of this activity was recovered in the "soluble" fraction (i.e. <5%). However, in this experiment enzyme assays were carried out at 35°C; nitrilase activity in the "soluble" fraction at this temperature was later found to be relatively unstable (Section 5.3.1). Fig. 5.6 may be used to calculate what the theoretical nitrilase activity at 35°C would have been if denaturation had not occurred. If the line representing nitrilase activity from 17°C to 25°C is extrapolated on to 35°C, it indicates that the required correction factor would be 7.2. Thus the percentage of nitrilase activity present in the "soluble" fraction may have been as high as 35%. If so the loss of total nitrilase activity during cell disruption would only have been approximately 27%, a figure more in line with the loss of amidase activity.

The loss of total nitrilase and amidase activities during this attempt to prepare a cell-free extract does not necessarily mean that the enzymes were denatured during the process. The enzymes in the "soluble" fraction may have inherently lower activities than the same proteins *in vivo*, under the standard assay conditions.

Although this method for preparing cell-free extracts of *Rhodococcus* sp. was potentially superior to sonication, it was not used further during this project because our own laboratories did not have the necessary facilities.

5.3. INVESTIGATIONS INTO THE OPTIMUM CONDITIONS FOR ASSAYING THE NITRILASE AND AMIDASE ACTIVITIES IN THE *RHODOCOCCUS* SP.

5.3.1. The Effect of Temperature.

In the first in this series of experiments the effect of temperature on

the nitrilase and amidase activities of the "soluble" and "particulate" fractions and of an intact cell suspension was studied. The appropriate fraction (1ml), plus 3mls buffer was equilibrated for 5 minutes at the required temperature before adding the substrate solution which had been similarly preadjusted to the desired temperature.

Fig. 5.6 shows that the amidase activity of both fractions and of the intact cell suspension, when measured over an incubation period of 5 minutes, increased with increasing temperature up to 65°C. At 75°C there was a marked reduction in amidase activity in all cases,especially in the "soluble" fraction.

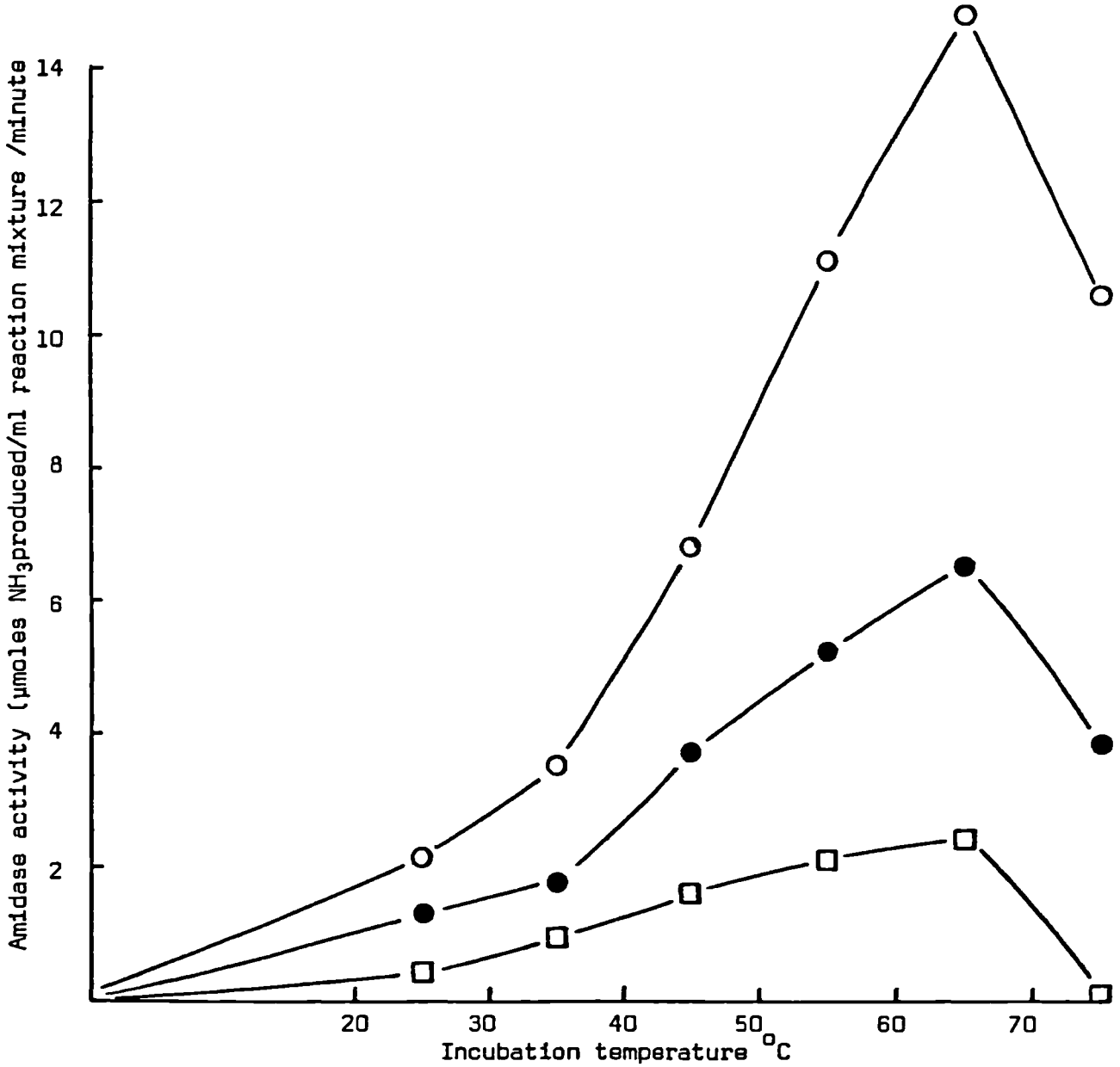
Nitrilase activity (Fig. 5.7) was far more heat labile than the amidase. The optimum temperature for assaying this activity in the "particulate" fraction and intact cell suspension was 35°C; at 45°C there was a very sharp reduction in activity while at 55°C no activity could be detected. Nitrilase in the "soluble" fraction was even more heat labile; activity was optimal at 25°C while at 35°C the activity had already decreased to only 18% of that measured at 25°C. Nitrilase activity could not be detected at all in the "soluble" fraction at 45°C.

In view of these results obtained for the nitrilase it was decided to continue assaying this activity at 25°C. In order to be able to compare nitrilase activity with amidase activity, the latter was also assayed at 25°C.

5.3.2. The Effect of Cold Storage on the Nitrilase and Amidase Activities of Intact Cells and Extracts of the *Rhodococcus* sp.

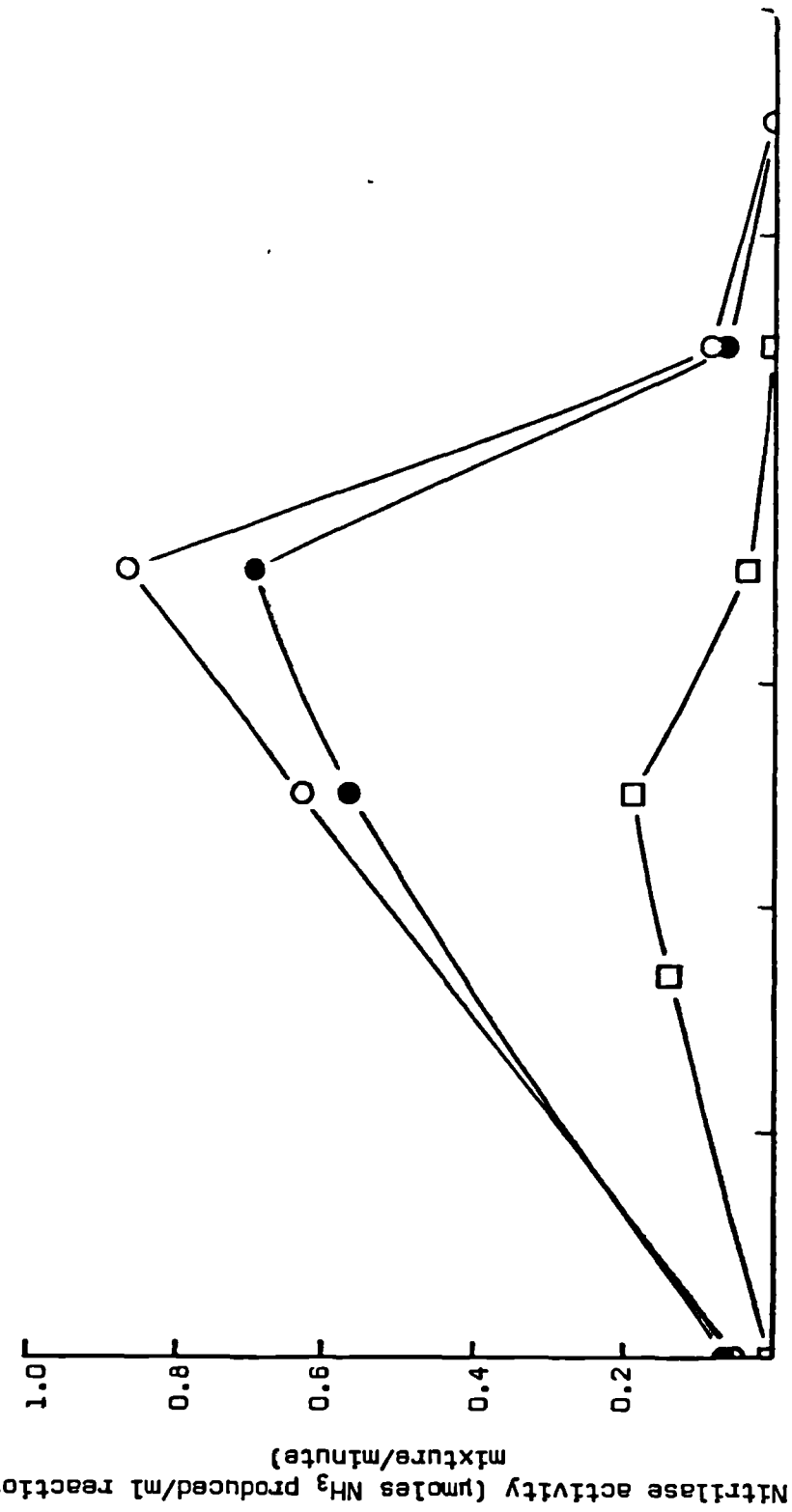
In the second in this series of experiments the long term stability of the nitrilase and amidase activities was investigated. Samples of "soluble" and "particulate" fractions and of an intact cell suspension were stored at 4°C and -20°C and the nitrilase and amidase activities determined at intervals over a period of 8 days.

FIGURE 5.6. The Effect of Temperature on the Activity of the *Rhodococcus* sp. Amidase.



The effect of temperature on the amidase activity of an intact cell suspension (O - O), "particulate" fraction (● - ●) and "soluble" fraction (□ - □) was investigated. Experimental details for growing the bacterium and for assaying and calculating the amidase activity were the same as those for Fig. 5.3 apart from the variable assay temperatures. The "soluble" and "particulate" fractions were obtained after sonicating the cell suspension for a total of 5 minutes (Section 2.4.1). Enzyme activities are expressed in comparative terms as described in Table 5.4.

FIGURE 5.7. The Effect of Temperature on the Activity of the *Rhodococcus* sp. Nitrilase.



The effect of temperature on the nitrilase activity of an intact cell suspension (O - O), "particulate" fraction (● - ●) and "soluble" fraction (□ - □) was investigated. Experimental details are similar to those described for Fig. 5.6.

Figs. 5.8, 5.9 and 5.10 show that the nitrilase and amidase activities were least stable in the "soluble" fraction at both temperatures. Freezing the "soluble" fraction at -20°C resulted in total loss of nitrilase activity (within experimental error). Amidase activity in the "soluble" fraction also decreased more rapidly when stored at -20°C compared to 4°C. Thus both enzymes in the "soluble" fraction appeared to be partially or completely denatured by freezing: their rapid loss of activity at 4°C may have been partly due to the presence of proteases in these crude extracts.

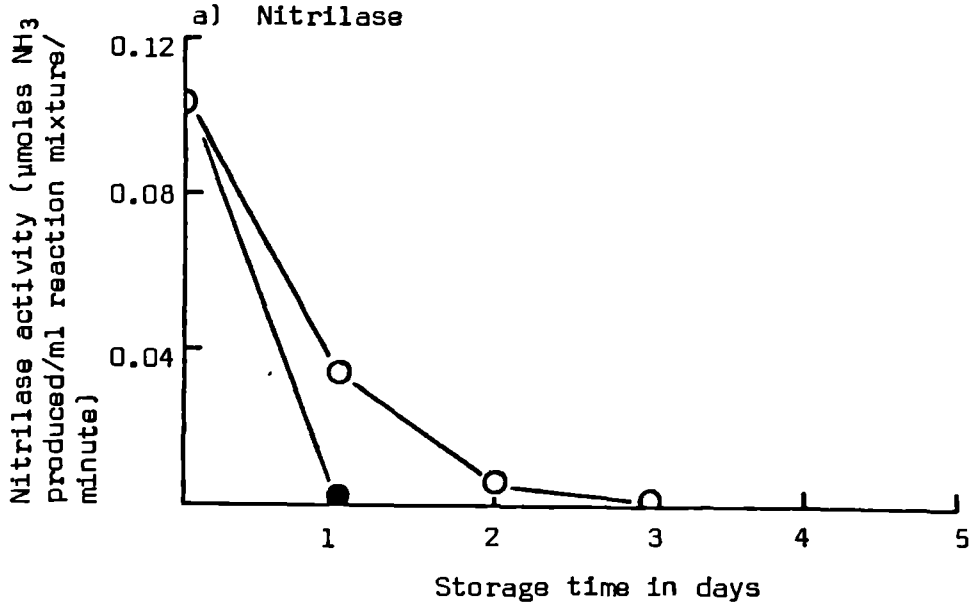
The stability of the nitrilase and amidase activities in the "particulate" fraction was similar to that for the intact cell suspension at both temperatures. Even here the act of freezing probably caused some denaturation but, after this, both activities remained relatively stable at -20°C.

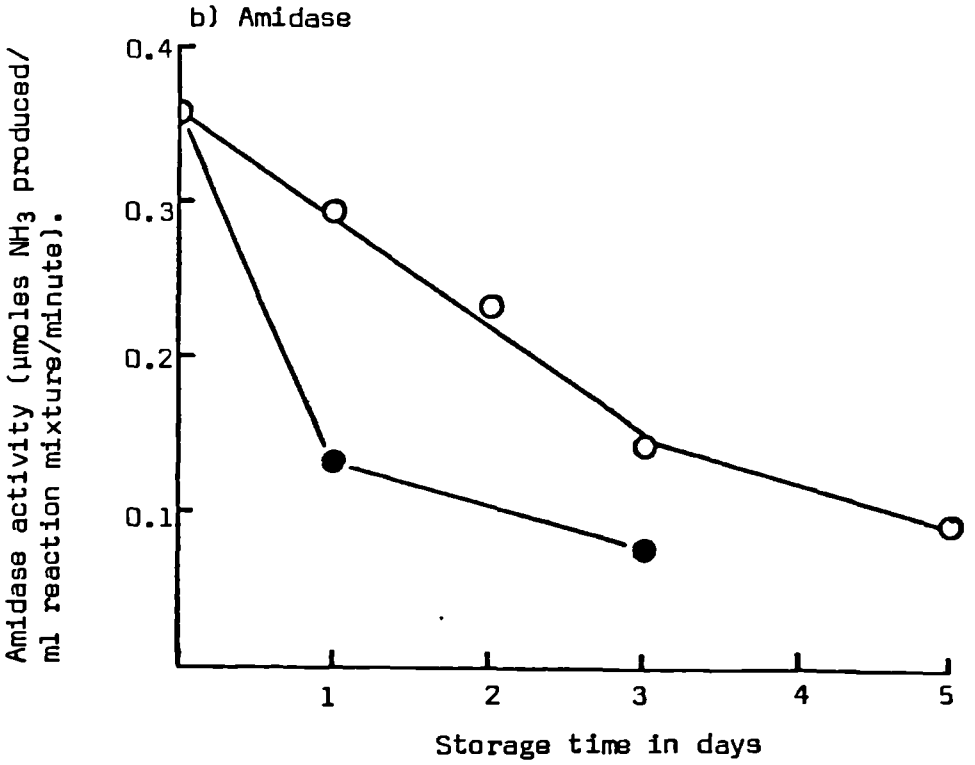
Since the enzyme activities in the "soluble" fraction decreased so rapidly with time, the effect of adding β-mercaptoethanol to the extracting buffer was investigated. β-Mercaptoethanol should help protect the enzymes from any oxidation which may occur during sonication and during storage. Ethylenediaminetetra-acetic acid-disodium salt (EDTA) was also incorporated into the extracting buffer since this was used in the preparation of extracts of *Nocardia rhodochrous* (N.C.I.B. 11216) active in hydrolysing benzonitrile (Harper, 1976). In addition, Jakoby & Fredericks (1964) found that EDTA increased acetamidase activity 2-3 fold when added to a purified preparation from *P. fluorescens*, an effect which could also be obtained with β-mercaptoethanol.

Fig. 5.11 and Table 5.9 show that similar results, for the "soluble" fraction, were obtained for this experiment compared to the last. Therefore, neither β-mercaptoethanol nor EDTA, when present at 2mM, had any effect on reducing the rate of decrease of nitrilase or amidase activities in the "soluble" fraction during storage at 4°C or -20°C.

FIGURE 5.8. The Effect of Storing the "Soluble" Fraction at $4^{o}C$ and $-20^{o}C$ on the Activity of the Nitrilase and Amidase.

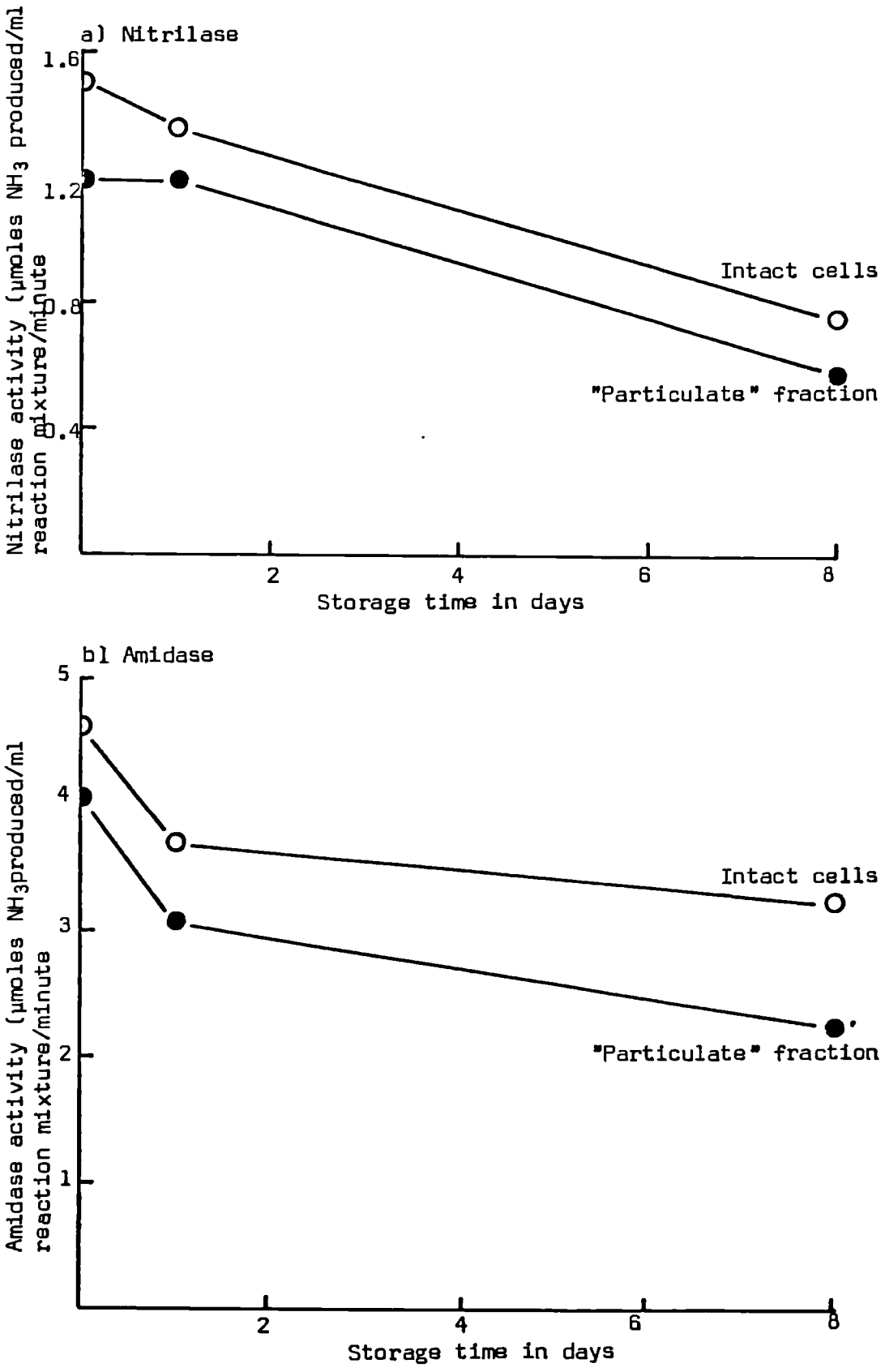
Figs. 5.8 - 5.10: The bacterium was grown and harvested as in Fig. 5.3. A portion of the cell suspension was sonicated for a total of 5 minutes and the "soluble" and "particulate" fractions separated as described in Section 2.4.1. Enzyme assays were performed as in Section 2.7.4 - modification 1. Ammonia was determined as detailed in Section 2.7.4 - "direct method" and activities calculated after subtracting the value for the appropriate no substrate control from experimental samples. The results are expressed in comparative terms as described in Table 5.4





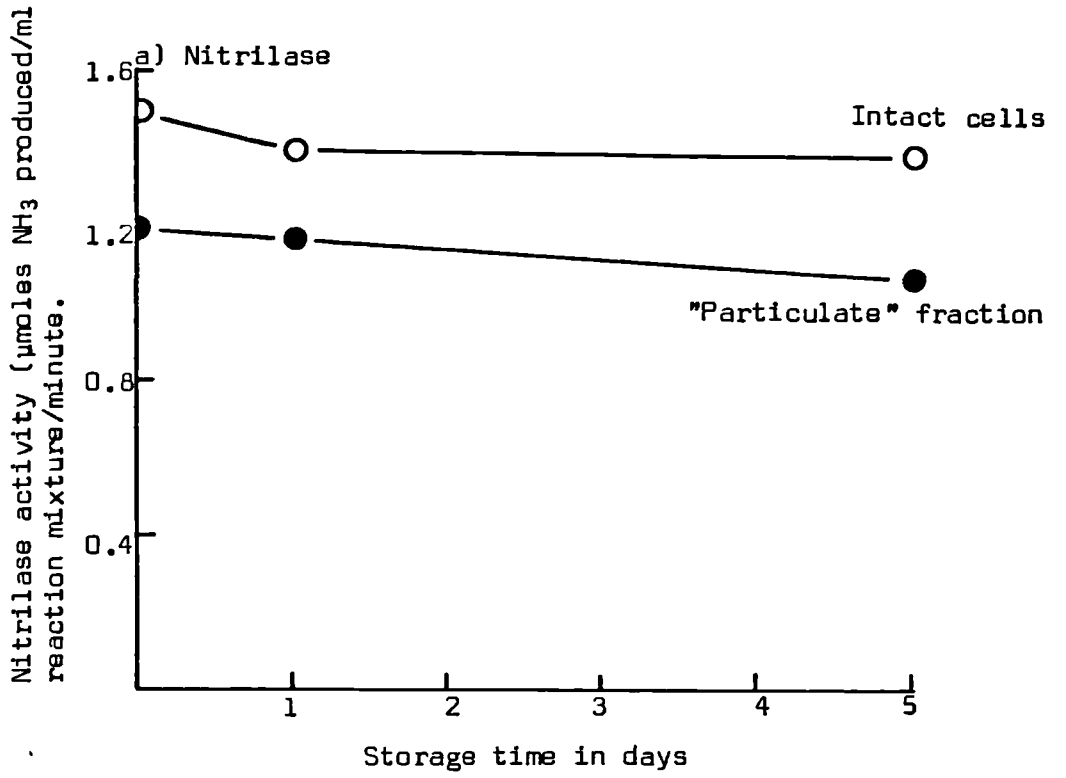
key: Enzyme activities in fractions stored at $4^{o}C$ (O - O) and $-20^{o}C$ (● - ●).

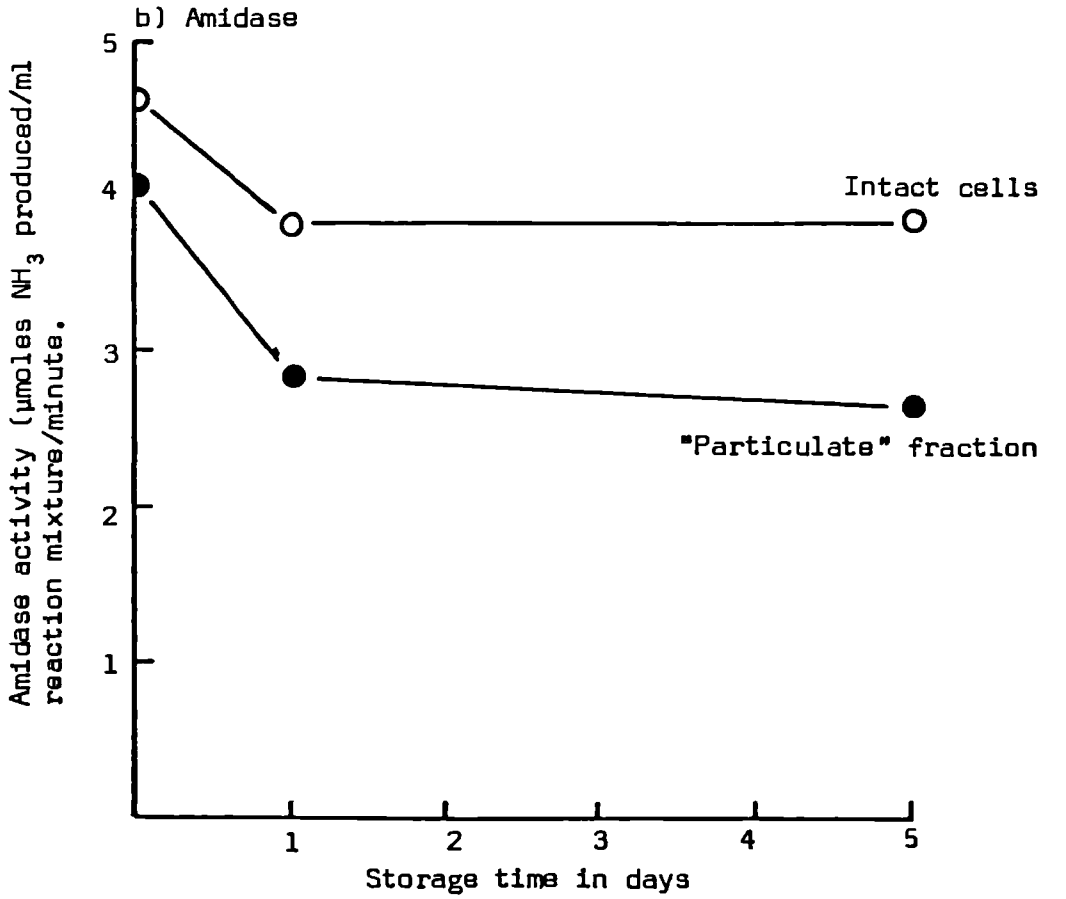
FIGURE 5.9. The Effect of Storing the "Particulate" Fraction and Intact Cell Suspension at 4°C on the Activity of the Nitrilase and Amidase.



For experimental details see Fig. 5.8

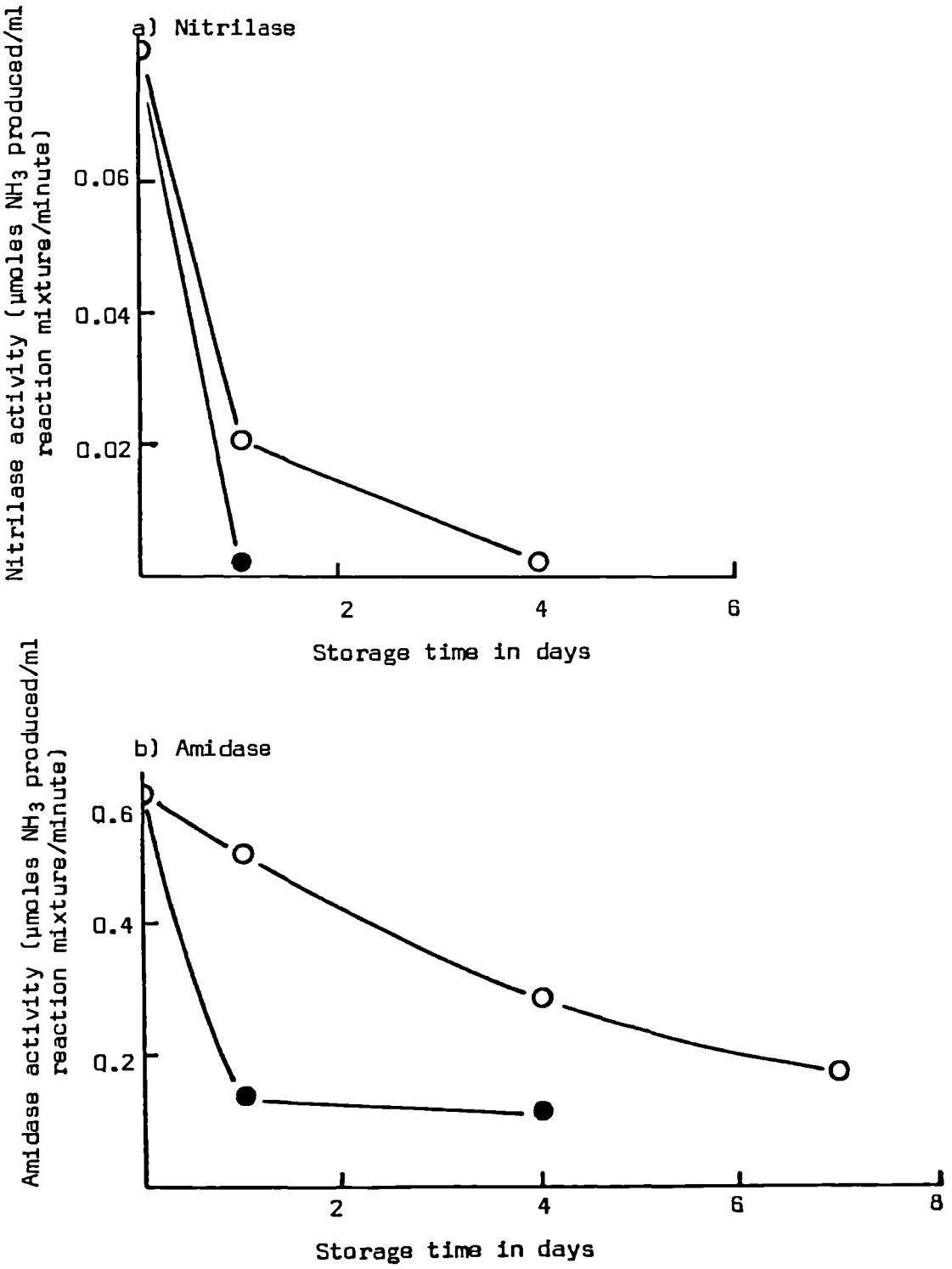
FIGURE 5.10. The Effect of Storing the "Particulate" Fraction and Intact Cell Suspension at -20°C on the Activity of the Nitrilase and Amidase.





For experimental details see Fig. 5.8

FIGURE 5.11. The Effect of Storing the "Soluble" Fraction at $4^{o}C$ and $-20^{o}C$ in the Presence of β-Mercaptoethanol and EDTA on the Activity of the Nitrilase and Amidase.



key: Enzyme activities in fractions stored at $4^{o}C$ (○ - ○) and $-20^{o}C$ (●-●)

Experimental details are exactly as described for Fig.5.8 except that the "soluble" fraction was prepared in the presence of 2mM β-mercaptoethanol and 2mM EDTA.

TABLE 5.9.

The % of Nitrilase and Amidase Activities Remaining in the "Soluble" Fraction After Cold Storage for 1 Day.

a) NITRILASE

Storage temperature	Day of Assay	% Activity remaining	
		Extract in buffer only	Extract in buffer plus β-mercaptoethanol and EDTA
4°C	0	100 %	100 %
	1	32.6 %	25.7 %
-20°C	0	100 %	100 %
	1	2.3 %	2.5 :

b) AMIDASE

Storage temperature	Day of Assay	% Activity remaining	
		Extract in buffer only	Extract in buffer plus β-mercaptoethanol and EDTA
4°C	0	100 %	100 %
	1	82.1 %	84.3 %
-20°C	0	100 %	100 %
	1	37.0 %	22.9 %

These values for the % enzyme activity remaining in the "soluble" fraction after cold storage for 1 day were calculated using the data presented in Figs. 5.8 and 5.11.

From these results it was obvious that experiments involving nitrilase in the "soluble" fraction would have to be carried out on the day of its preparation. Nitrilase activity in intact cell suspensions was also usually determined on the day of harvest although occasionally cell suspensions were kept for 1 day at $4^{o}C$ prior to use. Amidase activity was routinely assayed at the same time as the nitrilase.

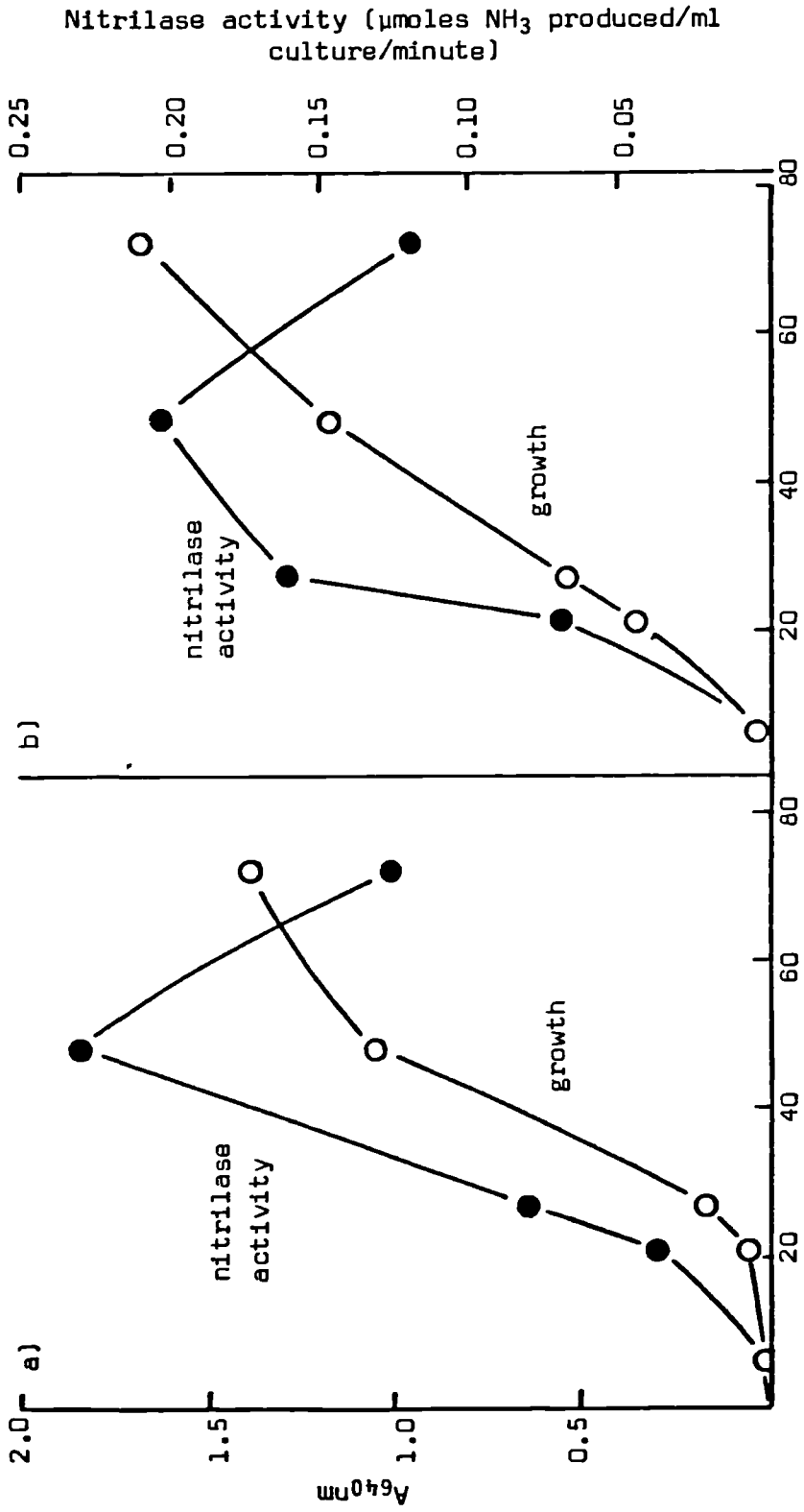
The low % recovery of the nitrilase and amidase activities in the "soluble" fraction obtained using the 75W sonicator available at the time, combined with the instability of the enzymes in this fraction, were serious disadvantages. Thus emphasis was placed on enzyme activities in intact cell suspensions in the majority of future experiments.

5.3.3. Nitrilase and Amidase Activities of the *Rhodococcus* sp. : Variation with Growth.

When the bacterium is grown on acetonitrile, presumably a proportion of it must be converted to acetate and ammonia prior to the onset of growth. Thus the nitrile and amide components may be rapidly depleted. If the nitrilase and amidase enzymes require induction by their substrates then such a depletion in the availability of inducer molecules may lead to a reduction in the synthesis of nitrilase and amidase by the culture at some stage during growth.

Nitrilase and amidase activities were therefore measured at different stages of growth to determine the optimum time to harvest cultures for future experiments. The bacterium was grown in m.m. of two different compositions, namely, BSM plus sodium acetate (0.5% w/v) and acetonitrile (0.1% v/v) and BSM plus acetonitrile (0.25% v/v) only. The variations in the total activities of the enzymes during growth on the two media are shown in Figs. 5.12 and 5.13.

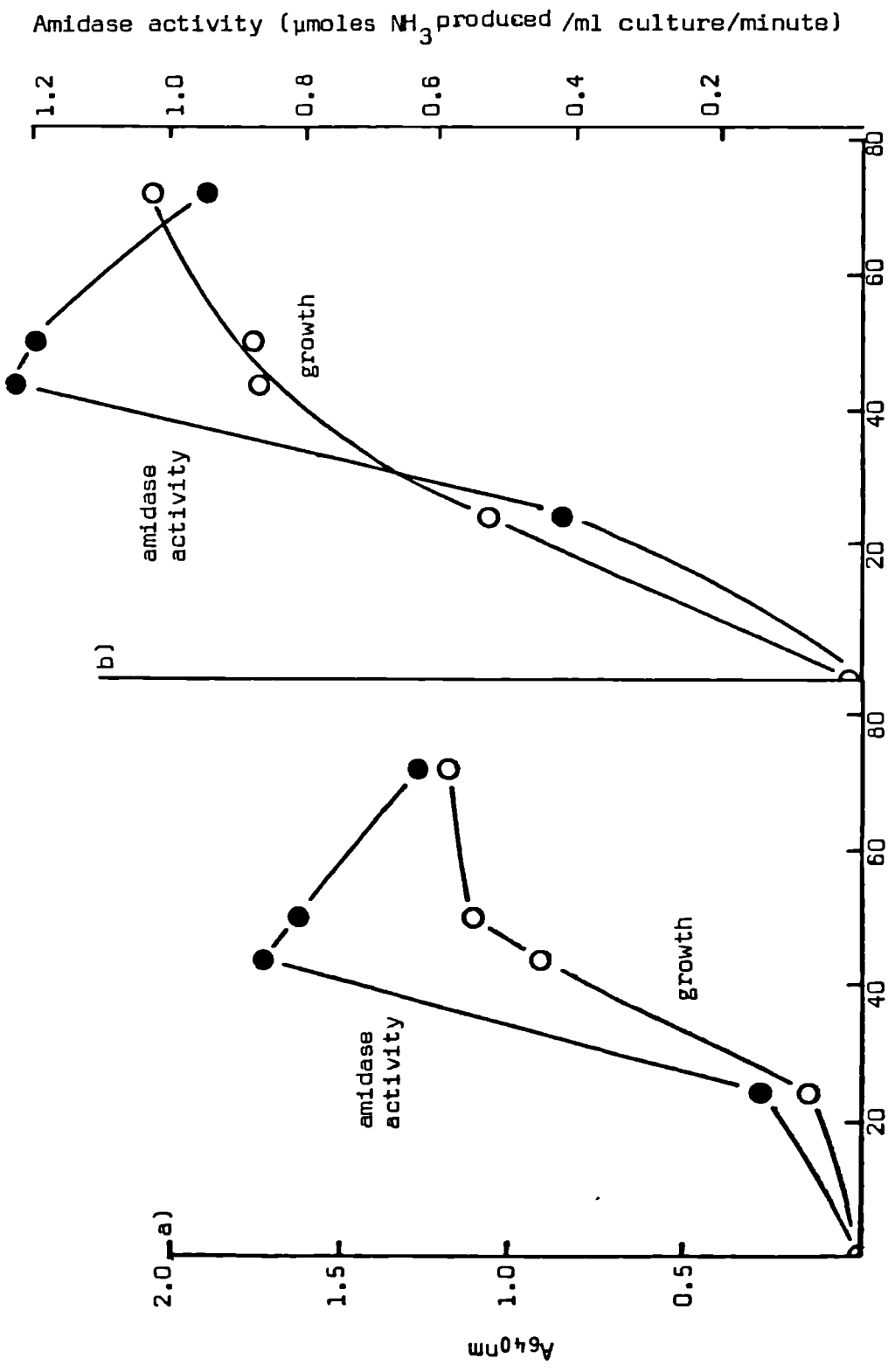
FIGURE 5.12. Nitrilase Activity of the *Rhodococcus* sp. During Growth.



a) Growth medium: BSM plus 0.25% (v/v) acetonitrile.
b) Growth medium: BSM plus 0.5% (w/v) sodium acetate and 0.1% (v/v) acetonitrile

Starter cultures (10mls) were inoculated from a plate as described in Section 2.2.3 and grown for 2 days. The test medium (99mls) was inoculated with 1ml of starter culture grown in the same medium. Test cultures were shaken at 25°C and 5ml aliquots removed at intervals to measure the A_{640} and nitrilase activity. For the enzyme assay, the sample was centrifuged and the cells resuspended in 0.1M sodium phosphate buffer (pH 7.0) and assayed essentially as described for Fig. 5.3. Reaction mixtures were incubated for 30 minutes and activities calculated after subtracting the value for appropriate boiled control from the experimental sample.

FIGURE 5.13. Amidase Activity of the *Rhodococcus* sp. During Growth.



a) Growth medium: BSM plus 0.25% (v/v) acetonitrile. b) Growth medium: BSM plus 0.5% (w/v) sodium acetate and 0.1% (v/v) acetonitrile.

Experimental details were the same as those for Fig. 5.12 except for the different enzyme assayed and shorter incubation times (15 minutes) for reaction mixtures.

In all cases the nitrilase and amidase activities initially increased with growth but decreased sharply when the growth rate began to decline. All four of these growth versus enzyme activity experiments were repeated with similar results. The greatest nitrilase activity (in terms of rate of NH_3 production/ml of culture) was measured for cells grown in the BSM containing acetonitrile (0.25% v/v) as the sole C and N source. However, for the amidase, higher activities were recorded from the cells grown in the medium containing acetate and acetonitrile. Nevertheless, the specific activities for both enzymes (measured when the total activity for each was highest) were greatest in the culture grown in BSM plus acetonitrile (0.25% v/v) only (Table 5.10).

Therefore, in all future experiments, except for some described in Chapter 6, the *Rhodococcus* sp. was grown in BSM plus 0.25% (v/v) acetonitrile only. Cultures were always harvested during the latter half of the exponential phase of growth. When the bacterium was grown on a larger scale, in 5 l batch culture, the growth curve given in Fig. 5.14 was obtained: from this it was decided to routinely harvest cultures, grown in this way, after 24 hours. The mean doubling time of the *Rhodococcus* sp. in this medium, calculated from the formula: $\frac{\log_e 2}{k}$,

(where k = relative growth constant) was 5.5 hours at 25°C .

5.3.4. The Effect of Substrate Concentration on the Acetonitrilase and Acetamidase Activities in an Intact Cell Suspension of *Rhodococcus* sp.

Effect of Acetamide Concentration

Kelly & Clarke (1962) found that for *P.aeruginosa*, "acetamide hydrolysis by whole bacteria was optimal ..... with an amide concentration of 0.2M;" as determined by the rate of ammonia production. Thus the effect of acetamide

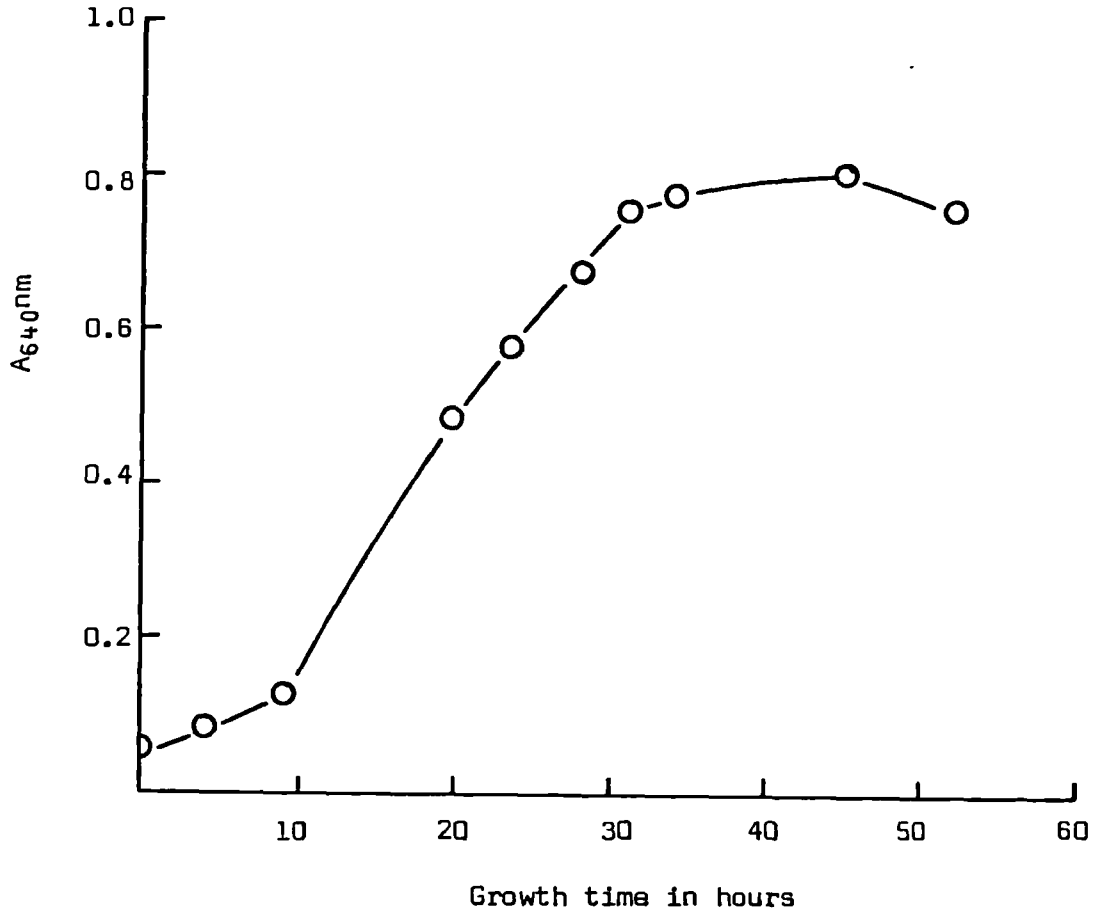
TABLE 5.10

Specific Activities of the Nitrilase and Amidase of Cells of *Rhodococcus* sp. Grown in Media Containing Acetonitrile With or Without Acetate

Medium	Assay	Specific activities (μmoles NH_3 produced/ml culture corresponding to 1 O.D. unit at 640nm/minute).
BSM plus 0.25% (v/v) acetonitrile only	Nitrilase	0.205, 0.218
	Amidase	0.893, 0.953
BSM plus 0.1% (v/v) acetonitrile and 0.5% (w/v) sodium acetate	Nitrilase	0.133, 0.173
	Amidase	0.649, 0.707

Specific activities were calculated from Figs. 5.12 and 5.13 by dividing the highest enzyme activities recorded in each culture by the corresponding A_{640} measurements. The duplicate results are from two completely independent experiments.

FIGURE 5.14. Growth of the *Rhodococcus* sp. in 5 *l* of BSM containing 0.25% (v/v) Acetonitrile as the Sole Source of C and N.



The bacterium was grown as described in Section 2.2.3 and Fig. 2.1. Samples were removed aseptically at intervals and the A_{640} recorded.

concentration on the amidase activity of an intact cell suspension of the *Rhodococcus* sp. was investigated.

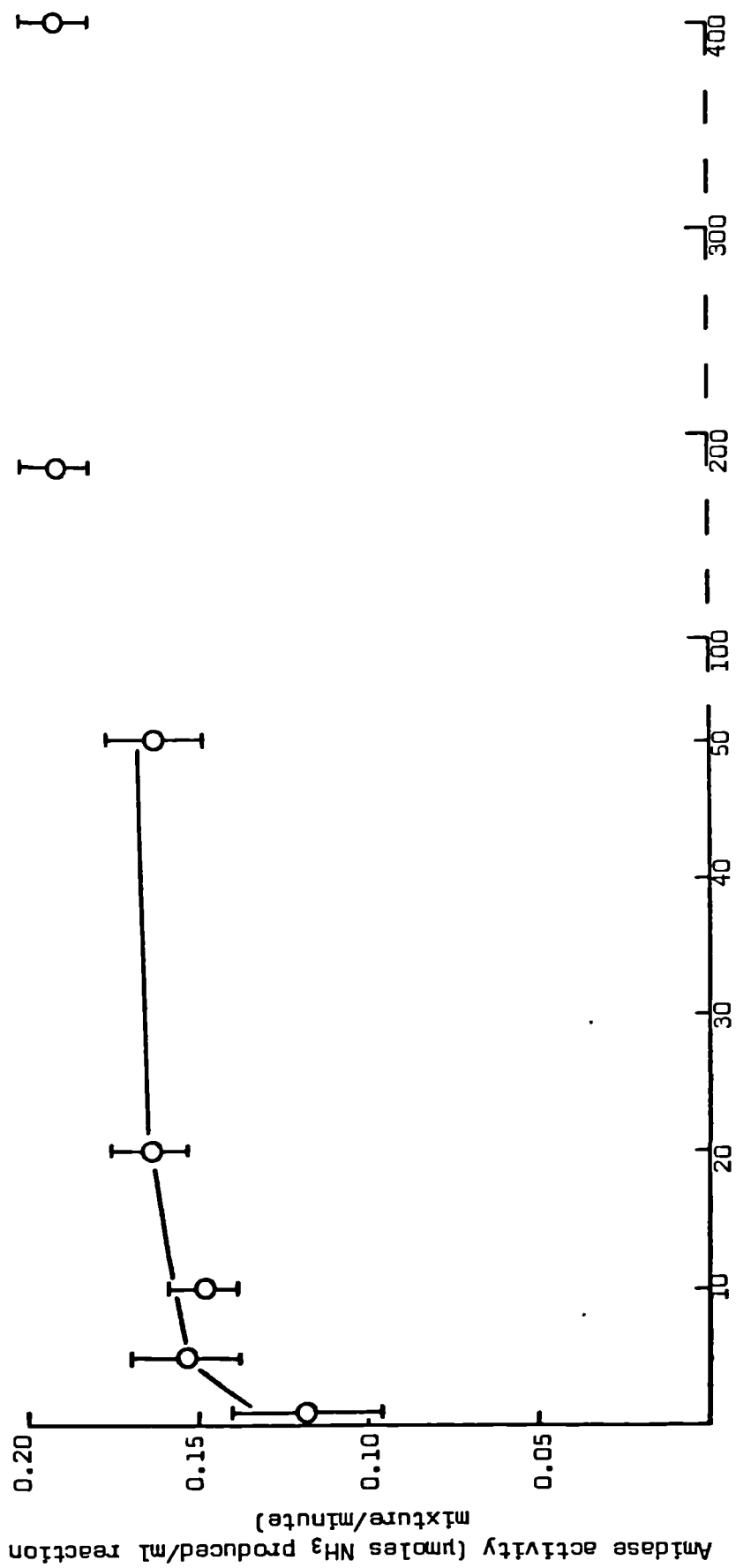
Acetamide concentrations in the reaction mixture ranging from 1-400mM were tested. Samples were taken for analysis at 0 and 10 minutes when the amide concentration was 50mM or greater. Since substrate exhaustion could cause a non-linear progress graph at low substrate concentrations, samples were taken for analysis at 0, 5, 10 and 15 minutes when the amide concentration was 20mM or less. In practice, when the initial concentration was 5, 10 or 20mM the rate of acetamide hydrolysis was constant during the 15 minute incubation period. When the amide concentration was only 1mM however, there was approximately 100% hydrolysis within 10 minutes. Thus the rate for this assay was calculated from the 5 minute sample which corresponded to approximately 60% hydrolysis.

From Fig. 5.15 it can be seen that the average rate of acetamide hydrolysis was greater in reaction mixtures containing the amide at an initial concentration of 200 and 400mM. However, although the average rate of acetamide hydrolysis was lower when present at 50mM, it was not significantly so. The rates at 5, 10, 20 and 50mM were also the same within experimental error. However, in reaction mixtures containing an initial amide concentration of 1mM the average rate of acetamide hydrolysis was only 62% of the maximum rate observed.

Thus it appears that the k_m for the acetamidase system in whole bacteria is less than 0.7mM. The quality of the data did not justify a more precise estimate.

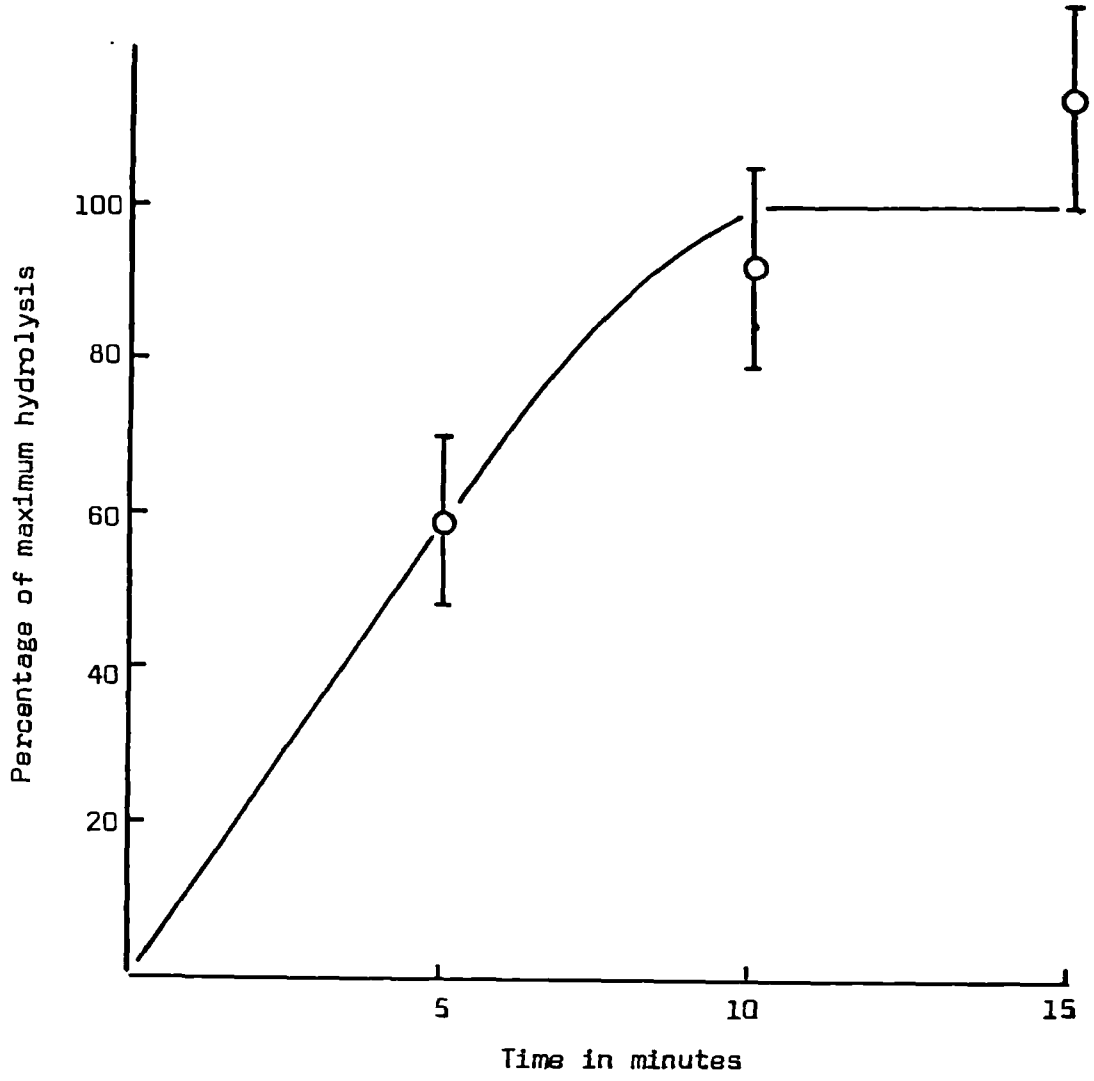
The hydrolysis of acetamide in reaction mixtures containing an initial amide concentration of 1mM was demonstrated to proceed to completion (Fig. 5.16). Thus the amidase not only hydrolyses acetamide quantitatively but the ammonia produced by the intact cells is all released into the medium.

FIGURE 5.15. Amidase Activity in Cells of the *Rhodococcus* sp.: Effect of Substrate Concentrations.



The bacterium was grown as for Fig. 5.14 and harvested and resuspended as in Section 2.7.4. The cell suspension was diluted 25-fold and assayed for acetamidase activity as in Table 7.3 - method 1 (micro-diffussion) except for the nature and variable concentration of the substrate. The difference between values obtained for the zero-time and 10 minute "experimental" samples was used to calculate amidase activity except when acetamide was present at an initial concentration of 1mM when the value for the zero-time sample was subtracted from the 5 minute experimental sample. Results are the average of • duplicate assays.

FIGURE 5.16. Acetamide Hydrolysis by Intact Cells of the *Rhodococcus* sp. : Percentage Conversion of Substrate to Ammonia with Time.



Ammonia formation in reaction mixtures containing 1mM acetamide and cells of the *Rhodococcus* sp. was followed at 5 minute intervals. Experimental details are the same as in Fig. 5.15.

Effect of Acetonitrile Concentration.

Acetontrile concentrations in the reaction mixture ranging from 5-300mM were tested. Samples were taken for analysis at 0 and 15 minutes. The rate of hydrolysis did not begin to decrease until the substrate concentration was lowered to 10mM (Fig. 5.17). When the substrate concentration was 5mM the rate was even lower but this was not due to substrate exhaustion since after 15 minutes incubation only 40% of the substrate had been hydrolysed to ammonia. Thus the optimum concentration of acetonitrile to use to assay nitrilase activity using intact cells was judged from this experiment to be 25mM or greater.

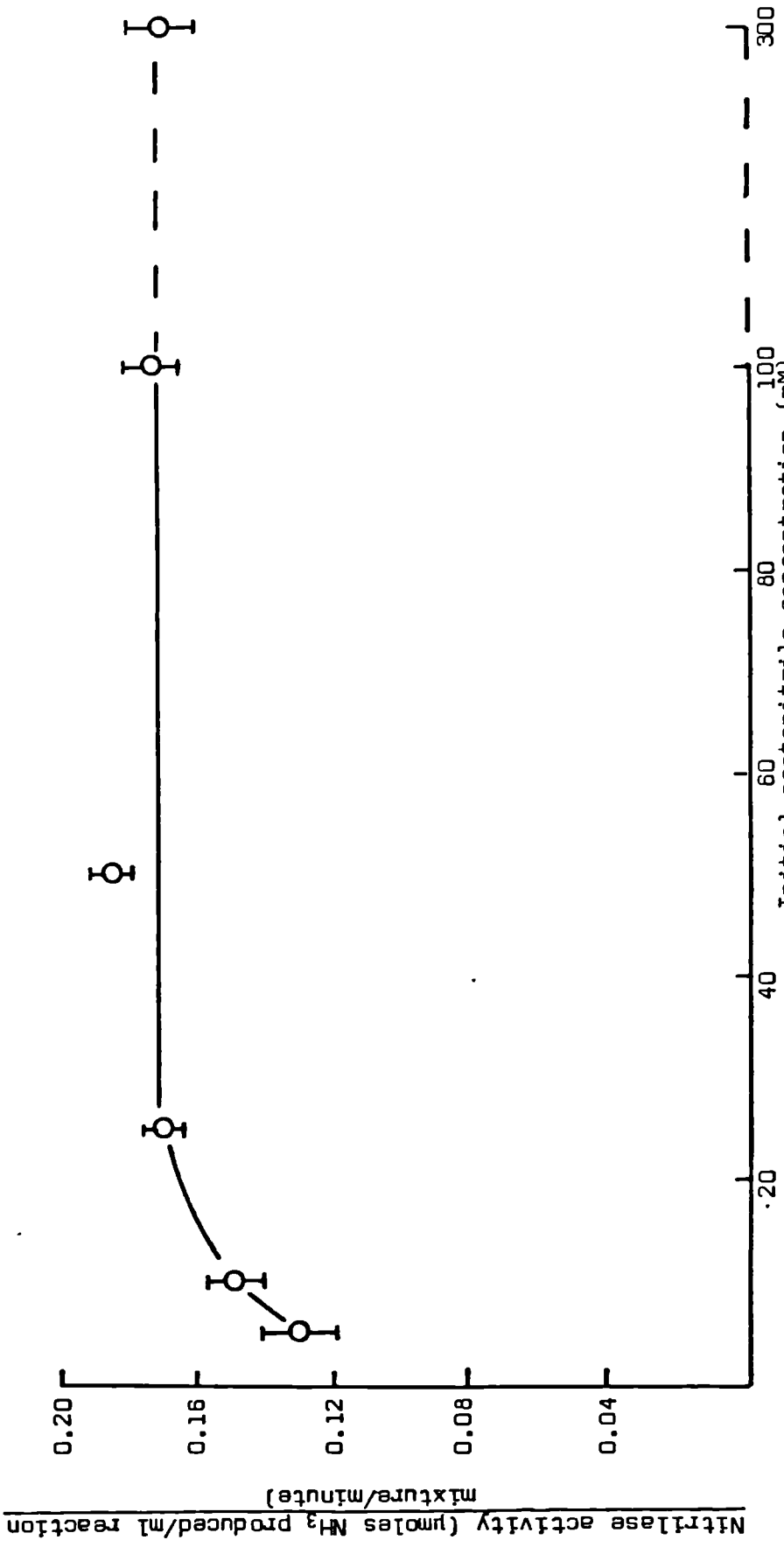
Using the values for the rates of acetontrile hydrolysis at the 3 lowest substrate concentrations, an approximate K_m was calculated for the nitrilase system in intact cells. From the Lineweaver-Burk plot shown in Fig. 5.18 the K_m for the coupled system converting acetonitrile to ammonia was calculated to be 1.5mM.

5.3.5. The Effect of the External pH on the Nitrilase and Amidase Activities in an Intact Cell Suspension of the *Rhodococcus* sp.

The effect of pH on both acetonitrile and acetamide hydrolysis by intact cells of the bacterium was investigated within the buffering range of orthophosphate.

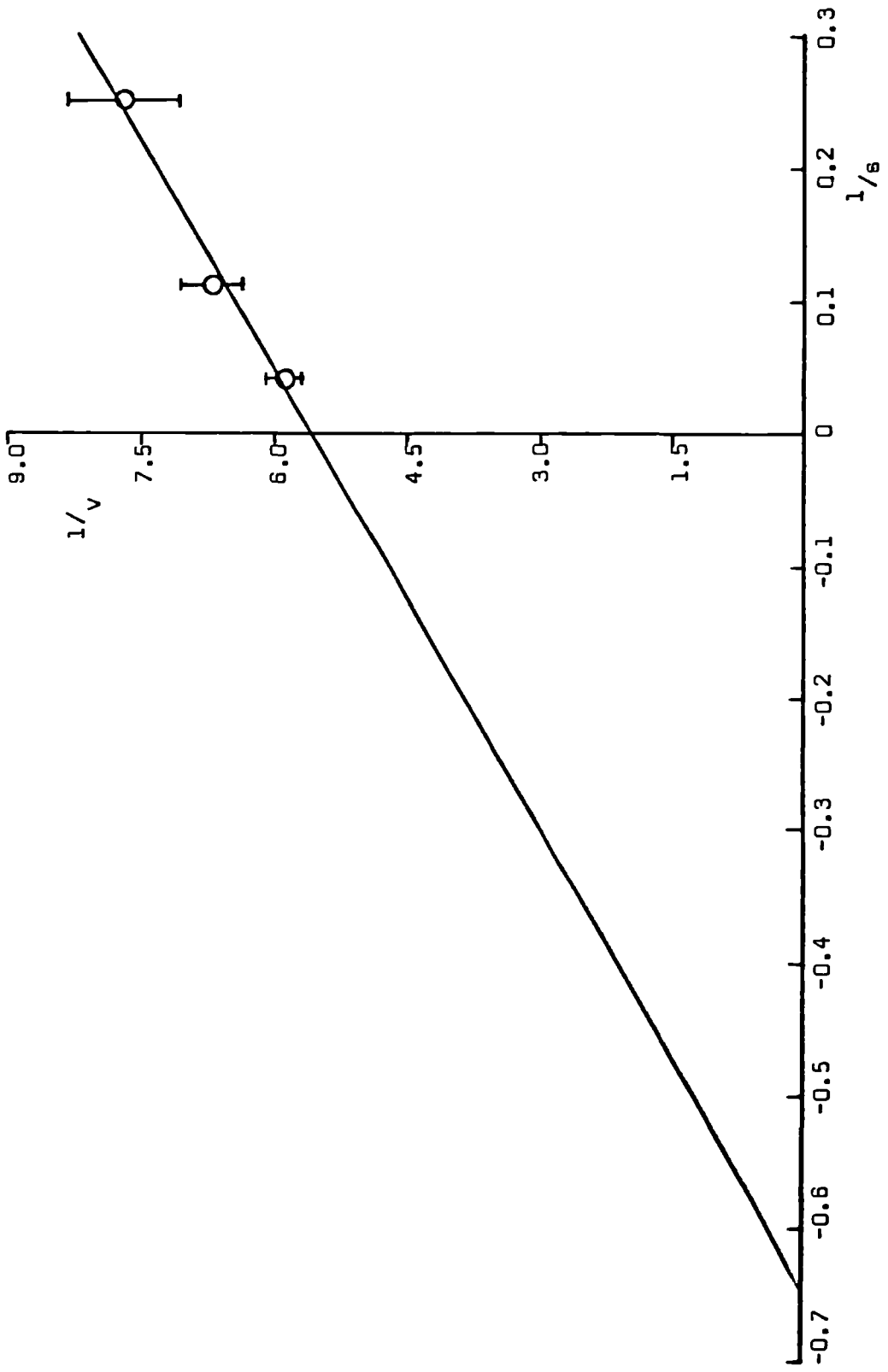
From Table 5.11 it can be seen that both activities remained fairly constant when the external pH was varied between 6.25 and 7.8. The maximum variation in amidase activity was only approximately $\pm$ 7% whereas for the nitrilase activity it was even lower, $\pm$ 4.4%. In a later experiment (Section 7.1.2) it was shown that even if a reaction mixture containing intact cells and acetamide was mixed with an equal volume of saturated K_2CO_3 solution, which resulted in the pH rising to approximately 11.6, some amidase

FIGURE 5.17. Nitrilase Activity in Cells of the *Rhodococcus* sp.: Effect of Substrate Concentration.



Experimental details were essentially the same as those described for Fig. 5.15 except for the nature of the substrate. Also the cell suspension was only diluted 3-fold prior to assay. The difference between values obtained for the zero-time and 15 minute experimental samples was used to calculate nitrilase activity. Results are the average of duplicate assays.

FIGURE 5.18. Determination of the Apparent K_m for Acetonitrilase Activity in Intact Cells of the *Rhodococcus* sp.



The inverse of the activities given in Fig. 5.17 for reaction mixtures containing initial acetonitrile concentrations of 5, 10 and 25mM (1/v) were plotted against the inverse of the average substrate concentration in the appropriate reaction mixture during the 15 minute incubation period ($^{1}/_{s}$).

TABLE 5.11

The Effect of the pH of the Medium on the Nitrilase and Amidase Activities of Intact Cells of the *Rhodococcus* sp.

Assay	pH of reaction mixture	Activity (nmoles NH_3 produced/ml reaction mixture/minute)
Nitrilase	6.28	64 ± 5
	6.55	67 ± 7
	7.10	67 ± 7
	7.43	61 ± 6
	7.83	65 ± 8
Amidase	6.25	138 ± 2
	6.55	131 ± 2
	7.10	136 ± 3
	7.40	122 ± 3
	7.80	141 ± 3

The bacterium was grown as for Fig.5.14 and harvested and resuspended as in Section 2.7.4. The cell suspension was diluted 100-fold prior to the amidase assay and 3.5-fold prior to the nitrilase assay. Reaction mixtures were composed of 1ml diluted cell suspension (in 0.05M sodium phosphate buffer, pH 7.0), 2mls buffer (0.2M sodium phosphate) ranging in pH from 6.0-8.0 and 1ml of substrate solution (0.2M acetonitrile or acetamide in D.W.). Samples were removed for ammonia analysis by the direct method (Section 2.7.4) after 0 and 10 minutes incubation at 25°C; the pH of reaction mixtures were also measured at 0 and 10 mins and found to be as stated and not to vary during the incubation period. Activities were determined after subtracting the value for the appropriate zero-time control from the 10 minute experimental sample. No substrate — and no enzyme controls were also carried out and values for these remained constant throughout the 10 minute incubation.

activity still remained.

However, in practice, since the nitrilase and amidase activities of the intact cell suspension did not appreciably increase or decrease by varying the external pH between 6.2 - 7.8 it is reasonable to suppose that the assays were being carried out at the effective pH optimum. In future experiments therefore, 0.1M sodium phosphate buffer (pH 7.0) continued to be used in assays involving nitrilase and amidase activities.

5.4. THE HYDROLYSIS OF ACETONITRILE TO AMMONIA BY INTACT CELLS OF THE *RHODOCOCCUS* SP: A TEST TO ESTABLISH THE AMOUNT OF AMIDE REMAINING IN THE REACTION MIXTURE.

It was important to check whether or not there was any significant accumulation of the intermediate, acetamide, during the coupled assay for measuring nitrilase activity. One method of determining the amount of amide remaining in the reaction mixture would be to terminate the reaction and then add a cell suspension of *Pseudomonas putida* PPE 1 (amidase positive) to hydrolyse any remaining amide to ammonia.

Initially however, before putting this idea into practice a few experiments on the *P. putida* organism were necessary.

Development of the *P. putida* Assay.

P. putida PPE 1 grown in a m.m. containing acetamide synthesises an aliphatic amidase (Clarke, 1972). Previous experiments (Section 4.1.1) had shown that this bacterium was incapable of growing in a medium containing acetonitrile as the sole source of C and N suggesting it is unable to synthesise a nitrilase. If so, suitably induced cells of *P. putida* could be used as a source of amidase for converting any acetamide in a reaction mixture to ammonia.

The growth of *P. putida* in m.m. containing acetamide was first followed in order to determine when to harvest cultures in future experiments (Fig. 5.19). It may very well be important to harvest these cells during the log-phase of growth since Kelly & Clarke (1962) found that for *P.aeruginosa* (PAC 1) more amidase activity was present in cells harvested just before the end of log-phase compared to that after the end of growth and the same was true of the *Rhodococcus* sp. (Section 5.3.3).

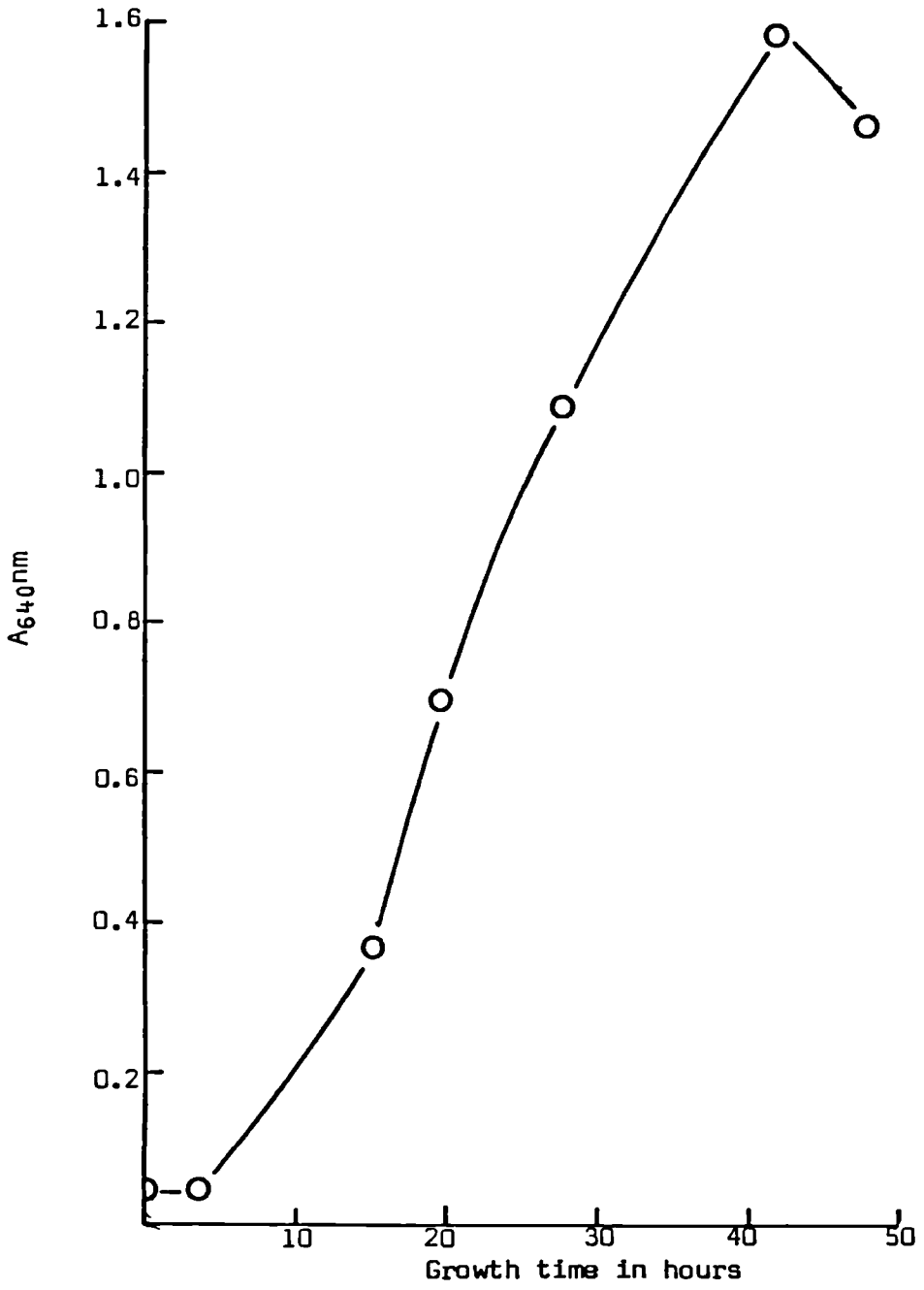
To determine whether or not *P. putida* PPE 1, grown in a m.m. containing acetamide, could hydrolyse acetonitrile, cells of the organism were assayed for nitrilase and amidase activity by the routine procedure (Section 2.7.4 - modification 2).

The rates of hydrolysis of acetonitrile and acetamide to ammonia, by the intact cell suspension, are given in Table 5.12. The value for the nitrilase assay was very low and close to experimental uncertainty. However, the results do demonstrate that cells of *P. putida* PPE 1 hydrolyse acetamide very rapidly. Therefore these cells were considered a suitable source of amidase to convert any acetamide in reaction mixtures, used to assay nitrilase activity, to ammonia.

To Establish the Amount of Amide Accumulation During the Coupled Assay.

The first part of the experiment corresponded to a typical coupled assay. Thus, 10mls of an intact cell suspension of the *Rhodococcus* sp. were incubated with 10mls 0.1M acetonitrile in buffer for 30 minutes at 25^{o}C. The reaction mixture was then centrifuged at 2,500g for 5 minutes and the supernatant fraction passed through a membrane filter (pore diameter 0.45µm) to ensure that all cells had been removed. To determine the concentration of acetamide in the filtrate the following reaction mixtures were set up:

FIGURE 5.19. Growth of *Pseudomonas putida* PPE 1 in Minimal Medium Containing 0.5% (w/v) Acetamide as the Sole Source of C and N.



The composition of the m.m. is given in Section 2.2.2. For the starter culture, 50mls of m.m. was inoculated with a loop of *P.putida* growing on a nutrient agar slope. After 2 days growth at 25°C with shaking, 5mls of starter culture was transferred to 95mls of fresh medium and growth followed by removing samples aseptically at intervals and recording the A_{640}.
The experiment was repeated and gave similar results.

TABLE 5.12

The Hydrolysis of Acetonitrile and Acetamide by an Intact Cell Suspension of Acetamide-Grown *Pseudomonas putida* PPE 1

Substrate	Assay	Ammonia analyses (A_{490})	Enzyme activity (µmoles NH_3 produced/ml of concentrated cell suspension/minute)
Acetonitrile	Experimental	0.783 ± 0.013	0.023 ± 0.005
	No substrate	0.725 ± 0.000	
Acetamide	Experimental	0.356 ± 0.006	103.5 ± 2.7
	No substrate	0.027 ± 0.002	

The bacterium was grown in m.m. containing acetamide (0.5% w/v) as the sole source of C & N (Section 2.2.2). Cultures (of total volume 500mls) were grown as described in Fig. 5.19 and harvested when the A_{640} was 1.08. Cells were washed in 0.1M sodium phosphate buffer (pH 7.0) and finally resuspended to 10mls. The experimental reaction mixture for assaying acetonitrile hydrolysis was composed of 0.5mls concentrated cell suspension, 0.5mls buffer and 1.0ml substrate (0.1M acetonitrile in buffer) and incubated for 60 minutes at 25°C. The experimental reaction mixture for assaying the amidase was composed of 0.005mls concentrated cell suspension, 0.995mls buffer and 1.0ml substrate (0.1M acetamide in buffer) and incubated for 30 minutes at 25°C. Reaction mixtures, for both assays, in which the substrate was replaced by buffer only were also analysed for ammonia concentration. The difference between the results obtained for the experimental and the no substrate control was used to determine enzyme activity. Ammonia concentration was determined by the "direct method" of nesslerisation (2.7.4). The values given are the means of duplicate assay.

(1) Experimental	:	3mls filtrate and 1ml of *P.putida* suspension.
(2) No filtrate control	:	3mls buffer and 1ml of *P.putida* suspension.
(3) No *p. putida* control	:	3mls filtrate and 1ml of buffer.
(4) *P. putida* + acetonitrile control	:	1ml *P. putida* suspension and 3mls acetonitrile solution (0.05M in buffer).

The reaction mixtures were incubated at 25°C for 30 minutes and then the concentration of ammonia in each was determined by nesslerisation as described in Section 2.7.4 - direct method. The results are given in Table 5.13 from which the following calculations were made:

(i) the apparent hydrolysis of acetonitrile in the presence of *P.putida* corresponded to an A_{490} increase of 0.009 $\pm$ 0.001 (reaction mixture 4 - 2);

(ii) the presence of *P. putida* cells gave rise to an increase in A_{490} of 0.075 $\pm$ 0.004 (reaction mixture 2 - R.B.);

(iii) therefore the corrected value for the experimental reaction mixture (1) was 0.526 - (0.009 $\pm$ 0.001 + 0.075 $\pm$ 0.004) $\doteq$ 0.442 $\pm$ 0.005.

Thus the concentration of ammonia in the filtrate after incubation in the presence of *P. putida* ($\equiv A_{490}$ of 0.442 $\pm$ 0.005) was the same as the concentration of ammonia in the filtrate after incubation in the absence of *P. putida* ($\equiv A_{490}$ of 0.447 $\pm$ 0.003, reaction mixture 3). Thus there was not sufficient amide in the filtrate to be hydrolysed by the cell suspension of *P. putida* to yield a detectable quantity of ammonia.

There are two remaining doubts however. First, any acetamide left at the end of the coupled assay might have been retained by the *Rhodococcus* cells. This is unlikely as discussed later in the next section of this Chapter.

TABLE 5.13

The Evaluation of Acetamide Accumulation During Acetonitrilase Assays of *Rhodococcus* sp. Cells : Measurement of Ammonia Released by *P. Putida* Amidase from Terminated Reaction Mixtures

Reaction mixture	Ammonia analyses (A_{490})
Reagent blank	0.019 ± 0.004
1 (Experimental)	0.526 ± 0.000
2 (- Filtrate)	0.094 ± 0.000
3 (- *P. putida*)	0.447 ± 0.003
4 (*P. putida* + acetonitrile)	0.103 ± 0.001

The suspension of *P. Putida* PPE 1 used here was potentially capable of releasing 1.38µmoles NH_3 from acetamide/ml cell suspension/minute, (determined by incubating a diluted suspension of the cells in the presence of 0.05M acetamide for 30 minutes at 25°C and calculating the amount of ammonia formed after subtracting the value for the no substrate control).

Secondly, over a period of approximately 2 years of regular sub-culturing there was a large reduction in the nitrilase activity of the *Rhodococcus* sp. culture but no simiar decrease in amidase activity. Initially, as determined in Section 5.1.2. the ratio of amidase to nitrilase activity was only 1 : 0.7. However, when the present experiment was carried out the ratio had fallen to 1 : 0.04. Therefore, this experiment has only validated the coupled assay for acetonitrilase in low nitrilase cells which were used in all later work described in this thesis. It is still possible that acetamide may have accumulated significantly in high nitrilase containing cells used earlier. Indeed, the earlier ratio of amidase to nitrilase activity determined (1 : 0.7) suggests that the amidase may well have once been the rate limiting step in the pathway for acetonitrile breakdown in cells of this *Rhodococcus* sp.

The observed reduction in nitrilase activity in the *Rhodococcus* culture with time could be explained if the nitrilase enzyme was coded for by extra-chromosomal DNA like that found in bacterial plasmids.

5.5. DISCUSSION

Some other workers have determined nitrilase activity in microorganisms by measuring ammonia production. Thus, Kuwahara *et al.*, (1980) used the technique to study nitrile and amide hydrolysis by extracts of *Fusarium solani* MN 7030, as did Harper (1977a & b) to investigate benzonitrilase activity in extracts of *F. solani* and *N. rhodochrous* N.C.I.B. 11216. The method has also been widely used to assay amidase activity by itself as in cells and extracts of *Mycobacterium smegmatis* (Draper, 1967) and *Lactobacillus* species (Hughes & Williamson, 1953) and in extracts of *Aspergillus nidulans* (Hynes & Pateman, 1970).

The fact that the nitrilase and amidase activities of the *Rhodococcus* sp. could be measured *in situ*, by using intact cells of the bacterium, is of considerable interest. It was certainly difficult to obtain more than a

small proportion of these activities in cell-free extracts. One possible explanation is that the enzymes are soluble but that cells of the *Rhodococcus* sp. were particularly resistant to disruption. If so the "particulate" fraction must have contained a large proportion of intact cells. Certainly the properties of the enzymes in the "particulate" fraction were very similar to those in intact cells.

An alternative possibility is that a small percentage of both enzymes may exist as a separate pool in the soluble form whereas the majority of the nitrilase and amidase may exist in a bound-form. The greater susceptibility of the nitrilase in the "soluble" fraction to high temperatures compared to that in the intact cells or "particulate" fraction would support this concept. However, this argument is not conclusive as dilution and other changes associated with releasing the enzymes into the medium could have a destabilising effect.

This ability to assay both the nitrilase and amidase *in situ* could indicate that they are surface enzymes, located possibly between the membrane and cell-wall, perhaps attached to the outer-face of the membrane itself. However, this is not necessarily so. Thalenfeld & Grossowicz (1976) found that the acetamidase activity of an unidentified thermophilic *Bacillus* sp. was the same whether whole bacteria or cell-free extracts were assayed. Yet after preparing protoplasts of the bacterium they found that the amidase was located within the protoplasts and not in the "periplasmic space" between the cell-wall and cytoplasmic membrane. It was also shown not to be attached to the membrane itself and was therefore considered to be cytosolic.

The nitrilase and amidase activities of *Brevibacterium* R312 have also been studied using intact cells (Arnaud *et al.*, 1976a,b and c).Both of these activities were attributed to soluble enzymes although their location was not investigated. Similarly,it was concluded that the acetonitrilase of

Nocardia rhodochrous LL100-21 was both soluble and intracellular since the activity was recovered in the supernatant fraction after centrifuging a sonicated suspension at approximately 35,000g (DiGeronimo, 1975).

When establishing the amount of amide remaining in reaction mixtures containing *Rhodococcus* cells and acetonitrile, after terminating the coupled assay, cells of *P. putida* were used as a source of amidase (see Section 5.4). Similarly, later in Chapter 6, *P. putida* was used to convert any acetamide formed from acetonitrile, by amidase negative *Rhodococcus* cells, to ammonia (and acetate).

When assaying nitrilase activity in intact cell suspensions by this method or when determining the amount of acetamide remaining in the nitrilase reaction mixture, the assumption is made that any acetamide formed as a result of acetonitrile hydrolysis by the *Rhodococcus* sp. will be released into the medium where it will be available for hydrolysis by the *P. putida* cells. This is not an unreasonable assumption since when *N. rhodochrous* LL100-21 (DiGeronimo & Antoine, 1976) and *C. nitrilophilus* nov. sp. C-42 (Mimura *et al.*, 1969) are grown in media containing acetonitrile, the amide is initially released into the culture filtrate.

The nitrilase activity of the *Rhodococcus* sp. was very heat labile, particularly in the "soluble" fraction. The optimum temperature for assaying the activity in this fraction was only 25°C. Similar observations were made by Arnaud *et al.*, (1977) for the nitrilase enzyme in the "soluble" fraction of *Brevibacterium* R312. They found that the optimum temperature was 35°C; at 40°C the nitrilase activity began to decrease while at 45°C a sharp decline in activity occurred. A partially purified preparation of the nitrilase from barley leaves, which converts indoleacetonitrile to indoleacetic acid, was also shown to be heat labile since inactivation of the enzyme began at 35°C (Thimann & Mahadevan, 1964).

The amidase activity of the *Rhodococcus* sp. was more tolerant of high temperatures; the activity in the "soluble" and "particulate" fractions and intact cell suspension continued to increase up to 65°C but decreased sharply at 70°C. The optimum temperature for assaying the amidase in cell-free extracts of *Brevibacterium* R312 was fairly comparable: maximum activity occurred at 70°C while at 80°C no activity remained (Jallageas *et al.*, 1978).

Denaturation studies for amidase activities in species of Mycobacteria revealed that the activity of a purified preparation of nicotinamidase from *M. avium* was completely lost after 15 minutes at 50°C (Kimura, 1959a). Similarly, heating a cell-free extract of *Mycobacterium* 607 at 50°C for 15 minutes abolished 80% of its amidase activity towards nicotinamide and benzamide while heating at 60°C resulted in a complete loss of these activities without affecting the formamidase. The latter activity was only destroyed after heating at 80°C (Nagayama *et al.*, 1961).

Even at low temperatures the *Rhodococcus* nitrilase, and to a lesser extent the amidase, were not very stable in the "soluble" fraction. On storing this fraction at 4°C, half the nitrilase and amidase activities were lost after approximately 18 hours and 62 hours respectively. At -20°C, the "soluble" fraction lost half its amidase activity after approximately 19 hours, whereas no nitrilase activity could be detected after freezing and thawing this fraction. Both enzymes were more stable when stored *in situ* as the cell suspension or in the "particulate" fraction compared to the "soluble" fraction.

The benzonitrilase from *N. rhodochrous* (N.C.I.B. 11216) was also exceedingly labile in the purified form with a half-life of 10 hours at 0°C and undergoing a 90% loss of activity on freezing (Harper, 1977a). When the enzyme was only partially purified however, the half-life at 0°C was extended to 4 days. The benzonitrilase from *Fusarium solani* was slightly more stable,

loosing half its activity after 48 hours at 1°C and the same proportion on freezing (Harper, 1977b). Again, partially purified preparations were more stable having a half-life of 7 days at 1°C.

The nitrilase in extracts of an unidentified *Pseudomonas* sp. responsible for hydrolysing ricinine was quite different to those discussed so far in that it was stable for weeks when stored in the frozen state (Hook & Robinson, 1964). However, ricinine nitrilase was extremely labile when extracts were stored at 0°C, half the activity being lost within 4-5 hours (Hook & Robinson, 1964).

The acetamidase in cell-free extracts of *Brevibacterium* R312 was more stable than that of the *Rhodococcus* sp.: only 10% of its activity was lost after 7 days at 4°C (Jallageas *et al.*, 1978). Cell-free extracts of *Mycobacterium smegmatis* lost only 40% of their original acetamidase activity after storage at -10°C for 3 months (Draper, 1967).

Turning to intact cells, nicotinamidase activity in suspensions of *Lactobacilli* was reduced by 15-25% after 7 days at 2°C (Hughes & Williamson, 1953): the corresponding figure for the acetamidase in *Rhodococcus* sp. was a 29% loss in activity after 7 days at 4°C.

Neither β-mercaptoethanol or EDTA protected the "soluble" fraction of the *Rhodococcus* sp. against loss of nitrilase or amidase activity when stored at either 4°C or -20°C. Similarly, the acetamidase activity in frozen extracts of *M. smegmatis* was not stabilised by *B-mercaptoethanol* (Draper, 1967) and also the extreme lability of ricinine nitrilase in extracts of the *Pseudomonas* was not reduced by EDTA or sulphydryl compounds (Hook & Robinson, 1964). However, as described previously (Section 5.3.2) these supplements have been successful in protecting some other nitrilase and amidase preparations.

When the *Rhodococcus* sp. was grown in medium containing acetonitrile as

the sole source of C and/or N both the nitrilase and amidase activities of the culture increased with growth but fell sharply once the growth rate began to decline. This was different to the pattern observed for amidase synthesis in *P. aeruginosa* growing in acetamide medium (Clarke, 1970). With cultures of this organism the plot of amidase specific activity versus growth was shown to be bi-phasic. As growth began the specific activity of the amidase rose but then fell again. This decrease in specific activity when the culture was still in its log-phase was thought to be due to the depletion of the inducer, acetamide, from the medium. When the *P. aeruginosa* culture was reaching stationary phase however, the specific activity of the amidase increased very rapidly and was attributed to its gratuitous induction by acetate. The amidase synthesised by cells of *P. aeruginosa* when growing in a medium containing either acetamide or ammonium acetate was shown to be identical (Kelly & Kornberg, 1964).

The apparent K_m for the amidase assayed in intact cells of *Rhodococcus* sp. was less than 0.7mM whereas the comparable K_m for the nitrilase coupled to this enzyme was 1.5mM. The K_m values for the acetamidase and aceto-nitrilase enzymes in a cell-free extract of *Brevibacterium* R312 were found to be 3.5mM and 25mM respectively (Jallageas *et al.*, 1978; Arnaud *et al.*, 1977). This value for the amidase K_m was fairly close to that reported for a purified preparation of acetamidase from *Pseudomonas fluorescens* for which the K_m was 5mM, (Jakoby & Fredericks, 1964).

The K_m for the purified nitrilase from *Nocardia rhodochrous*, which converts benzonitrile directly to benzoic acid and ammonia was determined by Harper (1977a) to be 4.0mM, whereas the purified enzyme from the fungus *Fusarium solani* carrying out the same reaction had a K_m of 0.039mM (Harper, 1977b).

Since intact cells of the *Rhodococcus* sp. were used to determine optimum

substrate concentrations, the apparent K_m values obtained can only be compared cautiously with the other values reported which were measured using cell-free systems. However, assuming that the *Rhodococcus* cells do not actively accumulate the substrate molecules within the cell, if indeed this is where the amidase and nitrilase are located, then both the aceto-nitrilase and acetamidase enzymes of the *Rhodococcus* sp. have a relatively high affinity for their substrates.

Kelly & Clarke (1962) found that for *P. aeruginosa*, "acetamide hydrolysis by whole bacteria was optimal at pH 7.2 .....", as determined by the rate of ammonia production. However, varying the pH of reaction mixtures between pH 6.2 - 7.8 had no appreciable effect on the nitrilase or amidase activities of intact cells of the *Rhodococcus* sp. This result could be interpreted in two ways. If the nitrilase and amidase enzymes of the *Rhodococcus* sp. are located in the cytosol, the pH of the external medium within the range studied may not have altered the intracellular pH and no variation in enzyme activity would be observed. Alternatively, if the enzymes were subjected to a variation in pH in these experiments then the observations would be indicative of the enzymes having broad pH optima.

A broad pH optimum (pH 4.5 - 8.0) for the deamidation of nicotinamide was recorded using intact cells of *Lactobacillus arabinosus* (Hughes & Williamson, 1953): the same was observed in extracts of acetone dried cells. In contrast, however, although extracts of *Mycobacterium* 607 hydrolysed formamide over a wide pH range (6 - 9), activity was optimal between 7-8 (Nagayama *et al.*, 1961). Similarly, acetamidase in extracts of *Brevibacterium* R312 was active between pH 5-8 with a pH optimum between 6 and 7 (Jallageas *et al.*, 1978). The purified acetamidase from *P. fluorescens* was usique in having two pH optima, one acidic and one basic (Jakoby & Fredericks, 1964).

As for nitrilases, a broad pH optimum was observed for the partially purified enzyme from barley *(Hordeum vulgare)* leaves which hydrolyse indole-

acetonitrile to indole acetic acid. Its activity was constant between pH 5.5 - 8.0 with no activity below pH 5.0 or above pH 9.5 (Thimann & Mahadevan, 1964). The purified benzonitrilase from *F. solani* also exhibited a fairly broad plateau of activity between pH 7.8 - 9.1 quite unlike the sharp pH optimum (pH 7.95 - 8.05) shown by the bacterial benzonitrilase from *Nocardia rhodochrous* N.C.I.B. 11216 (Harper, 1977b; 1976). Finally, the acetonitrilase in extracts of *Brevibacterium* R312 was active within the pH range 6 - 9 with optimum activity occurring at pH 7.0 (Ar naud *et al.*, 1977).

CHAPTER 6

STUDIES ON THE BIOSYNTHESIS OF THE NITRILASE AND AMIDASE ENZYMES IN THE *RHODOCOCCUS* SP.

In order to investigate the possible induction and repression of the nitrilase and amidase enzymes in the *Rhodococcus* sp., the effect of growth medium composition on enzyme activity was studied.

6.1. ENZYME ACTIVITIES OF CULTURES GROWN IN ACETONITRILE, ACETAMIDE AND ACETATE/$(NH_4)_2SO_4$ MEDIA.

The media had the following compositions:

(a) BSM containing 0.25% (v/v) acetonitrile

(b) BSM containing 0.25% (w/v) acetamide

(c) BSM containing 1% (w/v) sodium acetate and 0.1% (w/v) $(NH_4)_2SO_4$.

All cultures were harvested during the log-phase. To determine how long to grow the cultures in order to harvest during the log-phase, the growth of the bacterium in media b and c was studied first, (Fig. 6.1).

Intact cell suspensions were used throughout for the determination of enzyme activities. Nitrilase and amidase activities were present in cells grown in all three media (Table 6.1). Cells grown in the acetate/$(NH_4)_2SO_4$ medium had the lowest specific activities for both enzymes. The ratio between the nitrilase and amidase activities varied according to the medium employed.

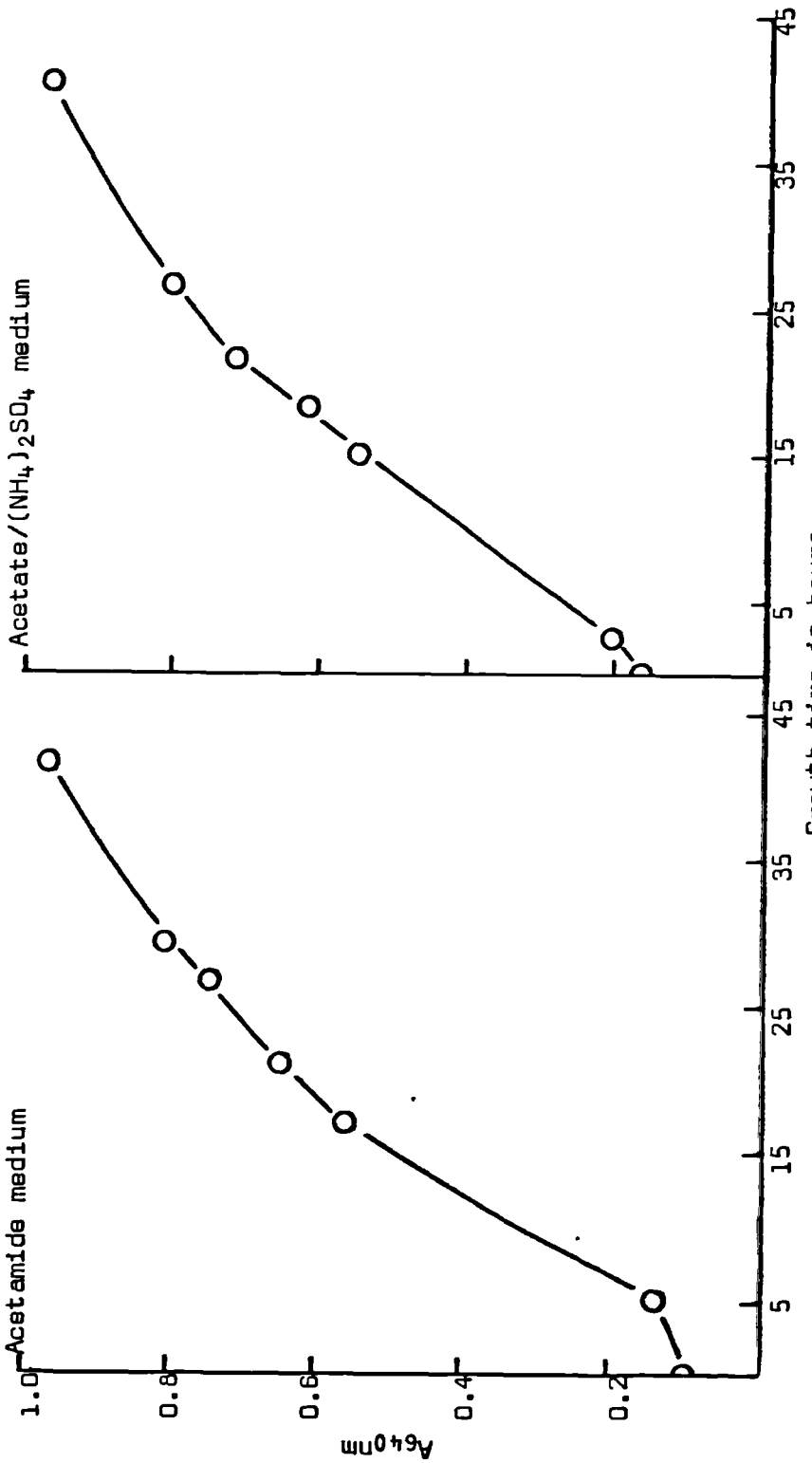
6.2. ENZYME ACTIVITIES OF CULTURES GROWN IN SUCCINATE/$(NH_4)_2SO_4$ MEDIUM.

The growth of the *Rhodococcus* sp. in BSM plus 1% (w/v) sodium succinate and 0.1% (w/v) $(NH_4)_2SO_4$ was studied first (Fig. 6.2).

6.2.1 Determination of Amidase Activity.

In the first experiment the culture was harvested when the A640 was 0.858 and amidase activity was measured using intact cells. Experimental details were the same as those for the acetamide grown cells (Table 6.1)

FIGURE 6.1. Growth of the *Rhodococcus* sp. in 5ℓ Batch Culture in Minimal Medium Containing Acetamide or Acetate/$(NH_4)_2SO_4$.



The bacterium was grown in 5 ℓ batch cultures as described in Section 2.2.3 and Fig. 2.1 except for the different nature or concentration of the C and N sources. Starter cultures were grown in media of similar composition to the 475Oml test medium. The exact compositions of the media are given in the text. Samples were taken aseptically at intervals and the A_{640}nm recorded.

TABLE 6.1

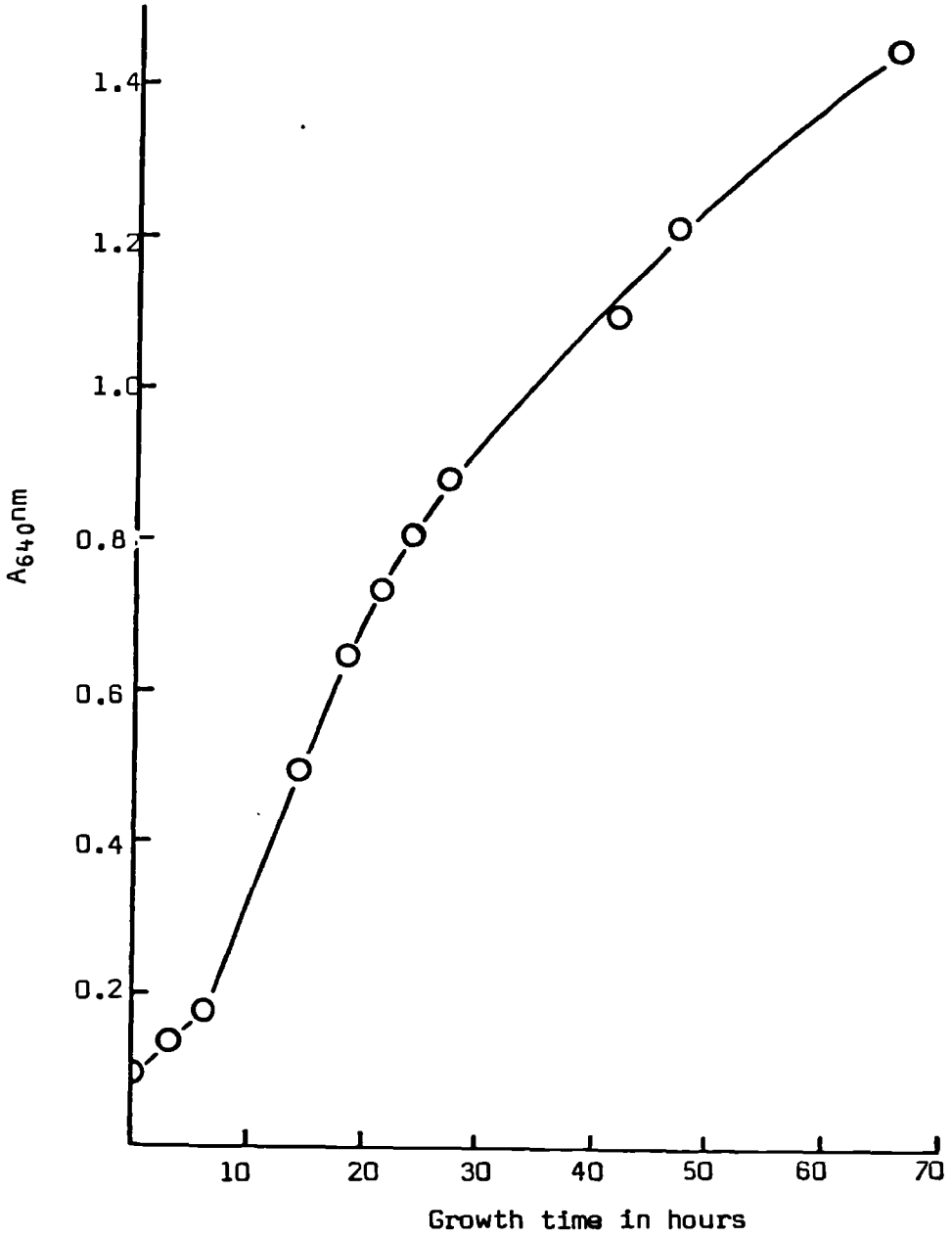
Nitrilase and Amidase Activities for Cells of the *Rhodococcus* sp. Grown in Acetonitrile, Acetamide and Acetate/$(NH_4)_2SO_4$ Media

C/N source(s) in the medium	Enzyme activity (nmoles NH_3 produced/mg dry wt. of cells/minute	
	Nitrilase	Amidase
Acetonitrile (0.25% v/v)	85.9 ± 3.1	2100 ± 200
Acetamide (0.25% w/v)	113 ± 4	1240 ± 40
Sodium acetate(1.0% w/v) + $(NH_4)_2SO_4$ (0.1% w/v)	17.9 ± 0.5	516 ± 18

Acetonitrile cultures. Exact details for obtaining these results are given in Table 7.7.

Acetamide and acetate/$(NH_4)_2SO_4$ cultures. The acetamide culture (5 *l*) was grown, harvested (when A_{640}nm was 0.512) and resuspended as described in Section 2.7.4. except that acetamide replaced the acetonitrile component in the growth medium. Nitrilase and amidase activities were assayed as described in Section 2.7.4 - Modification 2 and the ammonia concentration estimated after nesslerisation (Section 2.7.4 - "direct method"). Enzyme activities for cells grown in the acetate/$(NH_4)_2SO_4$ medium were determined in the same way except that the culture was harvested when the A_{640}nm was 0.567. Experimental reaction mixtures were incubated for 0 and 10 minutes and the difference between the two values obtained was used to calculate the enyme activity. Boiled and no substrate controls were also carried out; the values for these controls remained constant during the 10 minute incubation period. Results are the average of duplicate assays.

FIGURE 6.2. Growth of the *Rhodococcus* sp. in 5 *l* Batch Culture in Minimal Medium containing Sodium Succinate (1% w/v) and $(NH_4)_2SO_4$ (0.1% w/v).



The medium used for the starter cultures was of similar composition to the 4750 mls of test medium. The method for the growth of starter cultures and 5 *l* batch cultures is given in Sections 2.2.2, 2.2.3 and Fig. 2.1.

except that reaction mixtures were incubated for 30 minutes. Even with the concentrated cell suspension there was no significant difference between the absorbance values obtained, in duplicate, for the experimental, boiled control and no substrate control.

The second experiment was essentially the same except the cell suspension had been sonicated for 5 minutes immediately prior to use so that between 5 and 10% of the cells had been broken. Using the crude sonicated suspension, again there was no detectable hydrolysis of acetamide, thus confirming the negative results of the previous experiment and also demonstrating that they had not been due to permeability barriers. Thus, these results show that the amidase enzyme of the *Rhodococcus* sp. is not constitutive but one which requires induction.

6.2.2. Determination of Nitrilase Activity.

Acetonitrilase is routinely assayed by coupling it with the amidase normally present in the *Rhodococcus* sp. and determining the formation of ammonia. However, in this case endogenous amidase was absent and so the amidase activity of *P. putida* PPE 1 cells was used to couple the nitrilase activity (see section 5.4).

Both an intact cell suspension of *Rhodococcus* sp. and a suspension which had been sonicated for 5 minutes were investigated. The experimental reaction mixture consisted of 2.0mls of the concentrated cell or crude sonicated suspension of *Rhodococcus* sp., 1.0ml of a concentrated cell suspension of *P. putida* and 1.0ml of the substrate solution (0.2M acetonitrile in buffer). The 1.0ml suspension of *P. putida* cells was potentially capable of releasing 2500 nmoles NH_3/minute from acetamide (determined as described in the legend to Table 5.12) and so represented a large excess of amidase activity.

The results (Table 6.2) show that there was no detectable nitrilase activity in either intact cells or a sonicated suspension of *Rhodococcus* sp. which had been grown in succinate/$(NH_4)_2SO_4$ medium. This indicates that the nitrilase enzyme also requires induction. The results also confirm that acetamide grown cells of *P. putida* PPE 1 contain negligible nitrilase activity.

6.2.3. Induction of the Nitrilase and Amidase in *Rhodococcus* sp. Grown in Succinate/$(NH_4)_2SO_4$ Medium Plus Acetamide.

The synthesis of many inducible enzymes is repressed by glucose or other cell metabolites, e.g. β-galactosidase by glucose in *Escherichia coli*. This repression was known for a long time as "the glucose effect" but is more correctly described as "catabolite repression" (Magasanik, 1961). Brammar & Clarke (1964) found that the synthesis of the inducible aliphatic amidase of *P. aeruginosa* PAC 1 was repressed by tricarboxylic acid intermediates and metabolically related compounds in the growth medium. Thus in the previous experiments, succinate or one of its metabolites may have been acting as a repressor and not just as a non-inducer of the nitrilase and amidase in *Rhodococcus* sp. In order to investigate this possibility these enzymes were assayed in *Rhodococcus* cells grown in succinate/ammonium sulphate medium containing the known inducer acetamide.

The acetamide concentration was initially decided by reference to the work of Brammar & Clarke (1964). They had shown that amidase synthesis in *P. aeruginosa* PAC 1 growing in succinate/$(NH_4)_2SO_4$ medium could be detected after the addition of as little as 10^{-5}M acetamide. Upon addition of different concentrations of the non-substrate inducer N-acetylamide to *P. aeruginosa* growing in the same medium it was shown that there was no increase in the rate of amidase synthesis when the concentration of the

TABLE 6.2

Acetonitrile Hydrolysis by Succinate/$(NH_4)_2SO_4$ Grown Cells of *Rhodococcus* sp. in the Presence of Amidase Induced Cells of *P. putida* PPE 1

Form of the *Rhodococcus* sp.	Assay	A_{490}
Intact cells	"Experimental"	0.272 ± 0.003
	"No substrate"	0.254 ± 0.009
Crude sonicated suspension	"Experimental"	0.293 ± 0.005
	"No substrate"	0.294 ± 0.006

The *Rhodococcus* sp. was grown in 5 *l* batch culture and harvested when the A_{640} was 0.681. The cells were washed once in buffer and then resuspended to 100mls [section 2.7.4]. All reaction mixtures were incubated for 30 minutes and the ammonia concentration determined as for Table 6.1. Results are the average of duplicate assays. Other details are given in the text.

inducer was increased beyond 10^{-2}M suggesting that the system was saturated with inducer molecules at this point.

In the first experiment, acetamide was added to a culture of the *Rhodococcus* sp. after 10 hours growth in succinate/$(NH_4)_2SO_4$ medium, such that the overall acetamide concentration was 10^{-2}M (0.18% w/v). After a further 20 hours growth, the cells were assayed for amidase activity (Table 6.3). The amidase activity of cells grown in this way was only 17.6% of that for cells grown in medium with acetamide as the sole source of C and N (Table 6.1).

When the *Rhodococcus* sp. is growing in the succinate/$(NH_4)_2SO_4$ medium after the addition of acetamide, although amidase activity is not necessary for continued growth, the amide will be hydrolysed and metabolised as soon as some amidase has been synthesised. Therefore there are at least two possible explanations for the low specific activity of the amidase in cells from this culture. The first is that succinate or one of its metabolites is acting as a catabolite repressor of amidase synthesis. The second is that during the 20 hour growth period between adding the acetamide and harvesting the cells, the amide was hydrolysed and further metabolised to such an extent that few or no inducer molecules were left in the culture so that amidase synthesis ceased before growth terminated.

In the next experiment the following modifications were made: the amount of acetamide in the succinate/$(NH_4)_2SO_4$ medium was increased such that the overall concentration was 0.25% (w/v) and this was added to the medium at the same time as the starter culture. The culture was grown until the A_{640} was similar to that at the time of harvest for the culture grown in m.m. containing acetamide only, thus making the two sets of results comparable. This time both the nitrilase and the amidase activity was determined (Table 6.4).

The nitrilase and amidase activities in this experiment were approximately

TABLE 6.3

Amidase Activity in Cells of the *Rhodococcus* sp. Grown in Succinate/$(NH_4)_2SO_4$ Medium After Addition of Acetamide to the Exponentially Growing Culture

Assay	A_{490}	Amidase activity (nmoles NH_3 produced/mg.dry wt. cells/min.
"Experimental" zero-time	0.085 ± 0.001	
"Boiled control" "	0.052 ± 0.000	
"No substrate" "	0.047 ± 0.002	
"Experimental" 10 minutes	0.548 ± 0.013	218 ± 7
"Boiled control" "	0.052 ± 0.004	
"No substrate" "	0.044 ± 0.002	

The *Rhodococcus* sp. was grown in 5 *l* batch culture in m.m. containing 1% (w/v) sodium succinate and 0.1% (w/v) $(NH_4)_2SO_4$. After 10 hours growth, 20mls of a 29.5% (w/v) aqueous solution of acetamide was added aseptically by membrane filtration to the medium. After a further 20 hours growth the A_{640nm} was 0.834 and the cells were harvested, washed and resuspended as in the last experiment. Amidase activity was determined exactly as described in Table 6.1. The difference between values obtained for the zero-time and 10 minute "experimental" mixtures was used to calculate amidase activity. Results are the average of duplicate assays.

TABLE 6.4

Nitrilase and Amidase Activities in Cells of the *Rhodococcus* sp. Grown in a Medium Containing Succinate, $(NH_4)_2SO_4$ and Acetamide.

Enzyme assayed	Enzyme activity (nmoles NH_3 produced/mg dry wt. cells/minute)
Nitrilase	25.1 ± 0.7
Amidase	363 ± 4

A starter culture (250mls) was grown in BSM plus sodium succinate (1% w/v) and $(NH_4)_2SO_4$ (0.1% w/v) for 2 days at 25°C and used to inoculate 4750mls of similar medium but supplemented with 0.25% (w/v) acetamide. The culture was harvested when the A_{640}nm was 0.578 and the cells washed and resuspended as for the last experiment. Amidase activity was determined as in the last experiment; nitrilase activity was determined as described in Table 6.1.

22% and 29% respectively of those of cells grown in m.m. containing just acetamide. Since both the cultures being compared were grown in the presence of 0.25% (w/v) acetamide, the results obtained suggest that succinate or one of its metabolites is a catabolite repressor of nitrilase and amidase synthesis in the *Rhodococcus* sp.

6.3. DISCUSSION

Cells of the *Rhodococcus* sp. grown in the presence of either acetonitrile, acetamide or acetate/$(NH_4)SO_4$ contained nitrilase and amidase while those grown in succinate/$(NH_4)_2SO_4$ did not. Clearly both enzymes in the pathway for the degradation of aliphatic nitriles are subject to induction and/or repression in this *Rhodococcus* sp.. DiGeronimo & Antoine (1976) also found that the hydrolysis of acetonitrile by *N. rhodochrous* LL100-21 required prior induction. Fukuda *et al.*, (1971) however, demonstrated that cells of *Corynebacterium* HR3 could hydrolyse DL-α-aminopropionitrile to alanine whether grown in medium containing acetonitrile, glucose or succinate as a C source. Therefore here it was concluded that the enzyme(s) converting the aminonitrile to the corresponding amino acid was formed constitutively.

Acetonitrilase and acetamidase activities have been demonstrated in *Brevibacterium* R312 grown in a yeast carbon base medium containing either acetonitrile, acetamide or ammonium acetate as the sole source of N (Arnaud *et al.*, 1977). However, they concluded from the acetonitrilase results that since there was no significant difference in the specific activity measured for cells grown in the 3 different media, the enzyme was constitutive (Arnaud *et al.*, 1977; Jallageas *et al.*, 1980). However, the amidase activity was found to be 5-6 fold higher in cells from the aceto-nitrile or acetamide containing medium compared to cells grown in the presence of ammonium acetate. From these observations they concluded that

the amidase enzyme is apparently inducible (Jallageas *et al.*, 1978 & 1980). Nevertheless, cultures grown in complex YMPG medium (containing yeast and malt extract, bacto-peptone and glucose) were shown to hydrolyse a wide variety of nitriles and amides (Arnaud *et al.*, 1976a, b and c) suggesting that both enzymes are constitutive. Thus the regulation for the synthesis of these two enzymes in *Brevibacterium* R312 is not clear at present.

Some amidase enzymes, however, do appear to be constitutive. For example, Halpern & Grossowicz (1957) found that growth on nutrient agar did not prevent *Mycobacterium phlei* from hydrolysing formamide, acetamide, nicotinamide and several aminoamides. Also, Hughes & Williamson (1953) found that incorporating nicotinamide into the growth medium did not increase the level of enzyme responsible for its hydrolysis in *Lactobacillus arabinosus*.

When the *Rhodococcus* sp. was grown in acetonitrile medium both the nitrilase and amidase were induced but it is uncertain whether the nitrile or its hydrolysis product, acetamide, was the effective amidase inducer. The results already described showed that in the *Rhodococcus* sp., acetamide was a gratuitous inducer of the nitrilase enzyme and acetate a gratuitous inducer of both the nitrilase and amidase.

The gratuitous induction of an aliphatic amidase by acetate has also been demonstrated in *P. aeruginosa* (Clarke, 1970). However, this gratuitous synthesis only occurred at the end of the exponential phase of growth in acetate medium and no other aliphatic acid tested produced this effect. These observations were similar to those found for the amidase enzyme in *Mycobacterium smegmatis* (Draper, 1967). Although succinate-grown cells of this bacterium possessed a low amidase activity, it increased greatly when cells were grown in acetamide medium. *M. smegmatis* grown on acetate also showed enhanced rates of amide hydrolysis when compared to succinate-grown cells but propionate and butyrate, though able to support growth, were inefficient amidase inducers (Draper, 1967).

The reason for the gratuitous induction of the nitrilase and amidase in the *Rhodococcus* sp. is not understood. It has been suggested that sufficient acetamide can be formed chemically in a medium containing acetate and ammonia to induce the amidase in *P. aeruginosa* although this hypothesis has not been tested (personal communication by Brammar, W.J., cited by Clarke, 1970).

The results indicate that the nitrilase and amidase enzymes in the *Rhodococcus* sp. are probably subject to catabolite repression. This was shown to be a method of regulating the synthesis of the aliphatic amidase in *P. aerutinosa* and succinate was found to be one of the strongest repressors (Clarke, 1975). Also, Thalenfeld & Grossowicz (1976) demonstrated that the inducible amidase in a thermophilic *Bacillus* sp. was subject to catabolite repression by glucose.

When compounds are capable of acting as both a source of C and N, the enzymes responsible for their utilisation are sometimes subject to repression by both carbon and nitrogen metabolites,especially ammonia. This is true of the degradation of histidine in *P. aeruginosa* (Lessie & Neidhardt, 1967) and in *Aerobacter aerogenes* (Magasanik *et al.*, 1965). The most relevant enzyme regulated in this way is the amidase in *Aspergillus nidulans* (Hynes, 1970). It is possible that the reduction in the nitrilase and amidase activities in cells of the *Rhodococcus* sp. grown in medium containing succinate, ammonium sulphate and acetamide was caused by the repression of synthesis by ammonia.

However, if there was a high concentration of metabolites in cells of the *Rhodococcus* sp. growing in succinate medium (and if these metabolites had access to the enzymically active sites) then the observed reduction in nitrilase and amidase activities could also be explained by "catabolite inhibition". This is defined as, "the control exerted by glucose or other metabolites on the activity of certain enzymes", (Doelle, 1975). This type of control is analogous to the feedback inhibition of biosynthetic pathways.

CHAPTER 7

THE SPECIFICITY OF THE NITRILASE AND AMIDASE IN THE *RHODOCOCCUS* SP.

7.1. MODIFICATIONS OF THE NITRILASE AND AMIDASE ASSAYS.

Preliminary experiments revealed that some of the amides to be investigated as substrates for the amidase, caused a precipitate to form in the presence of Nesslers' reagent. It was therefore necessary to separate the ammonia released, as a result of nitrile or amide hydrolysis, from the reaction mixture. The obvious choice of technique to accomplish this separation was microdiffusion.

7.1.1. Development of the Microdiffusion Method for Ammonia.

To test the procedure detailed in Section 2.7.4, ammonium chloride samples in 0.08M sodium phosphate buffer, pH 7.0, were subjected to microdiffusion. Samples containing acetonitrile or acetamide (24.5mM) in the above buffer were also microdiffused to ensure that no appreciable spontaneous alkaline hydrolysis of these two compounds occurred.

Saturated potassium carbonate (K_2CO_3) solution was chosen as the alkali since this had been previously used by Kelly & Clarke (1962) to release ammonia from reaction mixtures containing amides. The sample (1.0ml) was placed in the vial and 1.0ml of the alkali solution added quickly. The cap, containing the acid trap, was immediately fitted onto the vial and microdiffusion was allowed to proceed for 2·5 hours.

The results (Table 7.1) show that there was no detectable hydrolysis of acetonitrile to yield ammonia and only a trace of ammonia was recovered from the vials containing acetamide. In addition the standard ammonium chloride samples gave consistent results and an almost linear calibration so the technique seemed suitable to determine ammonia production from acetonitrile and acetamide using intact cells of the *Rhodococcus* sp.

TABLE 7.1

Microdiffusion of Standard Samples

Sample	A_{420}nm after nesslerisation	A_{420}nm - R.B.
R.B.	0.025 ± 0.000	-
0.187mM NH_4Cl, (10µg/ml)	0.073 ± 0.000	0.048 ± 0.000
0.467mM NH_4Cl, (25µg/ml)	0.160 ± 0.004	0.135 ± 0.004
0.935mM NH_4Cl, (50µg/ml)	0.282 ± 0.006	0.257 ± 0.006
1.870mM NH_4Cl, (100µg/ml)	0.521 ± 0.002	0.496 ± 0.002
24.5mM acetamide (1.45 mg/ml)	0.033 ± 0.002	0.008 ± 0.002
24.5mM acetonitrile (1.00mg/ml)	0.014 ± 0.001	<R.B.

Samples were subjected to microdiffusion followed by nesslerisation as described in Section 2.7.4. - indirect method. Other details are given in the text. Results are the average of duplicate analyses.

7.1.2. Development of a Method for Terminating the Amidase Activity in Intact Cells of the *Rhodococcus* sp.

Termination with K_2CO_3.

The acetamidase activity of intact *P. aeruginosa* cells was inactivated when the reaction mixture was mixed with an equal volume of a saturated solution of K_2CO_3 (Kelly & Clarke, 1962). Thus it was decided to try this method for terminating the amidase activity in intact cell suspensions of the *Rhodococcus* sp.

After 0 and 10 mins. incubation, 1.0ml of reaction mixture was added directly to an equal volume of saturated K_2CO_3 and microdiffused for 2 hours. Boiled and no substrate controls were also carried out. The results (Table 7.2) indicate that the zero-time experimental sample contained large quantities of ammonia that were not present in the corresponding boiled or no substrate controls. Thus the amidase activity in intact cell suspensions of the *Rhodococcus* sp. could not be completely stopped by saturated K_2CO_3 solution.

Termination by centrifugation.

Aliquots of the reaction mixture (of similar composition to the previous experiment) were removed after 0, 10, 20 and 30 minutes and centrifuged immediately for 5 minutes at 2,500 g. Samples of the supernatant fractions were then microdiffused and the ammonia determined by nesslerization as before.

Although centrifugation of the reaction mixture prior to microdiffusion was comparatively successful in terminating the amidase activity, the progress curve, done in duplicate, was fairly erratic. The cells of the *Rhodococcus* sp. did not form a very firm pellet after centrifugation at 2,500 g, so

TABLE 7.2

The Use of Saturated K_2CO_3 Solution to Terminate Amidase Activity in Cells of the *Rhodococcus* sp.

Assay	Incubation time in minutes	A_{420nm}
Experimental	0	0.845 ± 0.005
Boiled control	0	0.034 ± 0.004
No substrate control	0	0.028 ± 0.005
Experimental	10	0.955 ± 0.075
Boiled control	10	0.037 ± 0.002
No substrate control	10	0.037 ± 0.010
R.B.		0.024 ± 0.000
NH_4Cl standard (0.935mM)		0.255 ± 0.000

The bacterium was grown in 5 l BSM plus 0.25% (v/v) acetonitrile and harvested and resuspended as described in Section 2.2.3 and 2.7.4. The concentrated cell suspension was diluted 50-fold and incubated with acetamide at a final concentration of 0.05M, the reaction mixture being otherwise as detailed in Section 2.7.4 - modification 1. After microdiffusion, ammonia concentrations were determined by nesslerisation as described in Section 2.7.4 - indirect method. Results are the average of duplicate assays.

variable quantities of cells were probably present in the supernatant fractions. These cells would continue to hydrolyse acetamide during the microdiffusion step and were probably responsible for the inconsistencies observed.

Termination with $AgNO_3$

Since the last method for terminating the amidase activity in intact cell suspensions was not very satisfactory it was decided to try and abruptly stop the activity by the addition of Ag ions.

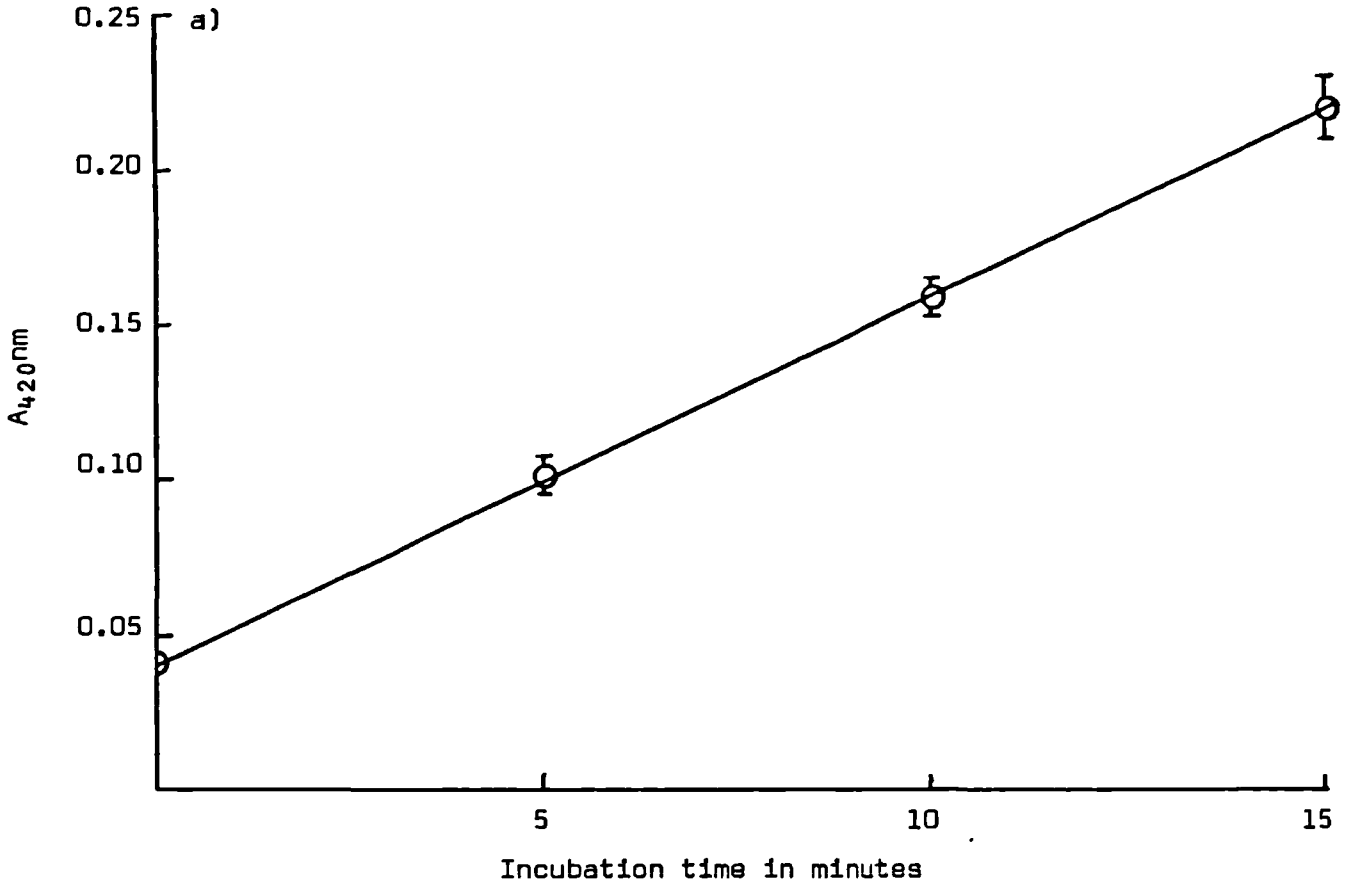
Other methods which would also be likely to terminate the activity, e.g. boiling or membrane filtration were considered less suitable. In the former case, boiling the reaction mixture would probably result in some loss of ammonia from solution and there would be a large increase in the amidase activity before being denatured. Membrane filtration would require a preliminary centrifugation of the reaction mixture, in experiments where concentrated cell suspensions were used and in addition some compounds to be tested, e.g. acrylonitrile, would react with the membrane.

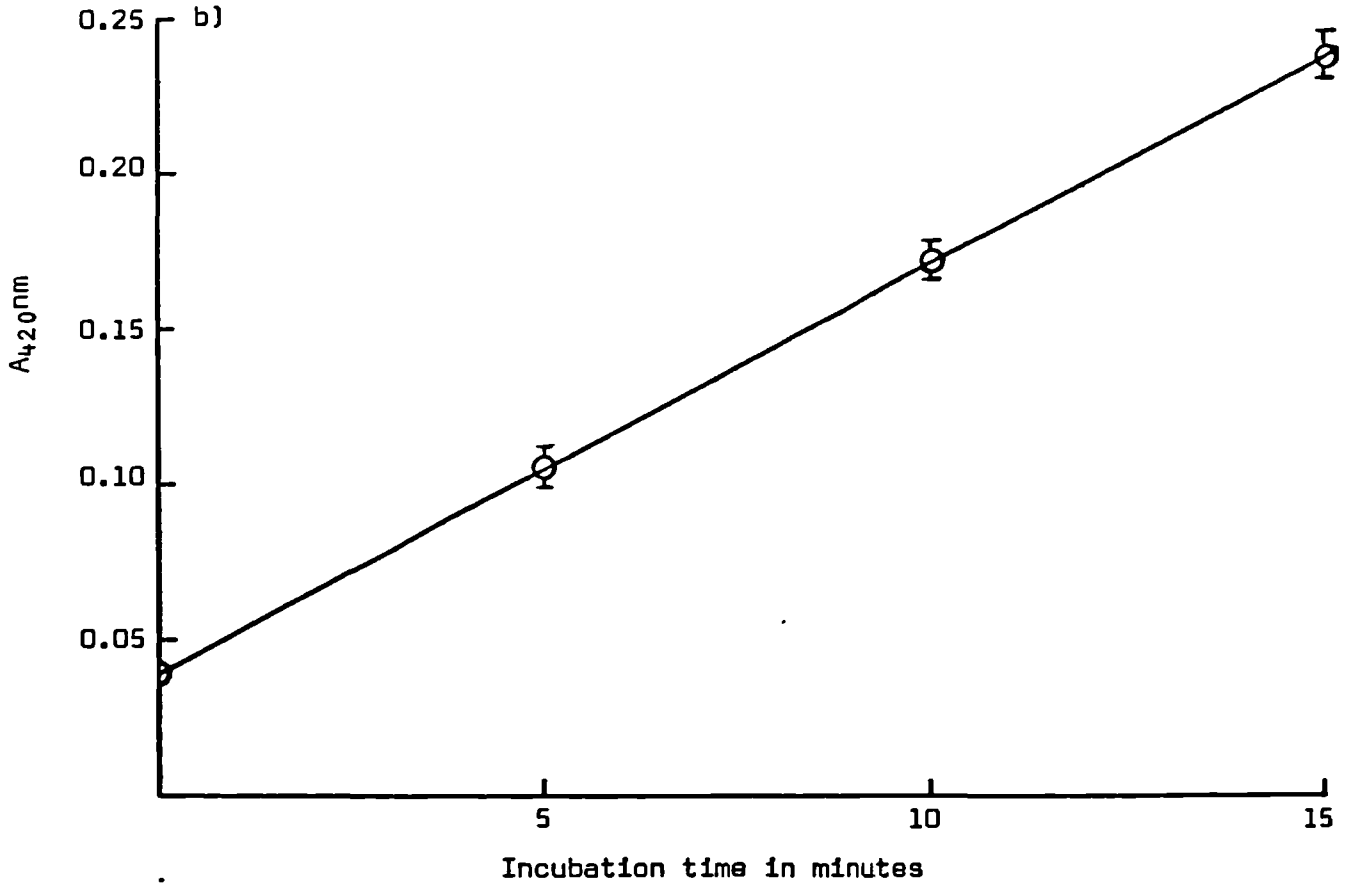
For the Ag ion termination method, 1ml aliquots of the reaction mixture were removed at intervals and added directly to 1ml of a 20mM solution of $AgNO_3$ in D.W. The mixture was shaken and when all samples had been treated thus, 1ml of each was subjected to microdiffusion.

The progress curve for the experiment, done in duplicate, (Fig. 7.1) was linear with time during the 15 minute incubation period. The absorbance for the zero time experimental sample was greater than that for the corresponding boiled control indicating there was a lag period before the Ag ions stopped the amidase activity. However, since both progress curves were linear, this lag period must have been constant for each of the experimental samples taken. One possible explanation for this lag period could be that

FIGURE 7.1. Acetamidase Activity in Cells of the *Rhodococcus* sp. Termination with Silver Ions.

The two progress curves (a and b) represent the results from two independent experiments. Reaction mixtures were of similar composition to the previous 2 experiments except the concentrated cell suspensions were only diluted 25-fold prior to assay. Details of the preparation of samples for microdiffusion are given in the text; ammonia production was determined as described in Section 2.7.4 - indirect method. Each progress curve is a plot of the results obtained for duplicate experimental assays less the values for the boiled controls which did not vary with time.


a)
0.25
0.20
0.15
0.10
0.05
A_{420nm}
5
10
15
Incubation time in minutes


b)
0.25
0.20
0.15
0.10
0.05
A_{420nm}
5
10
15
Incubation time in minutes

the Ag ions require a finite time to reach the site of the amidase enzyme.

This technique was next applied to assay acetonitrilase activity using intact cells of the bacterium. This preliminary experiment was necessary since the concentration of cells in the reaction mixture would be greater when the substrate was acetonitrile than when it was acetamide. It was therefore important to ensure that the same concentration of Ag ions would be sufficient for terminating the coupled assay.

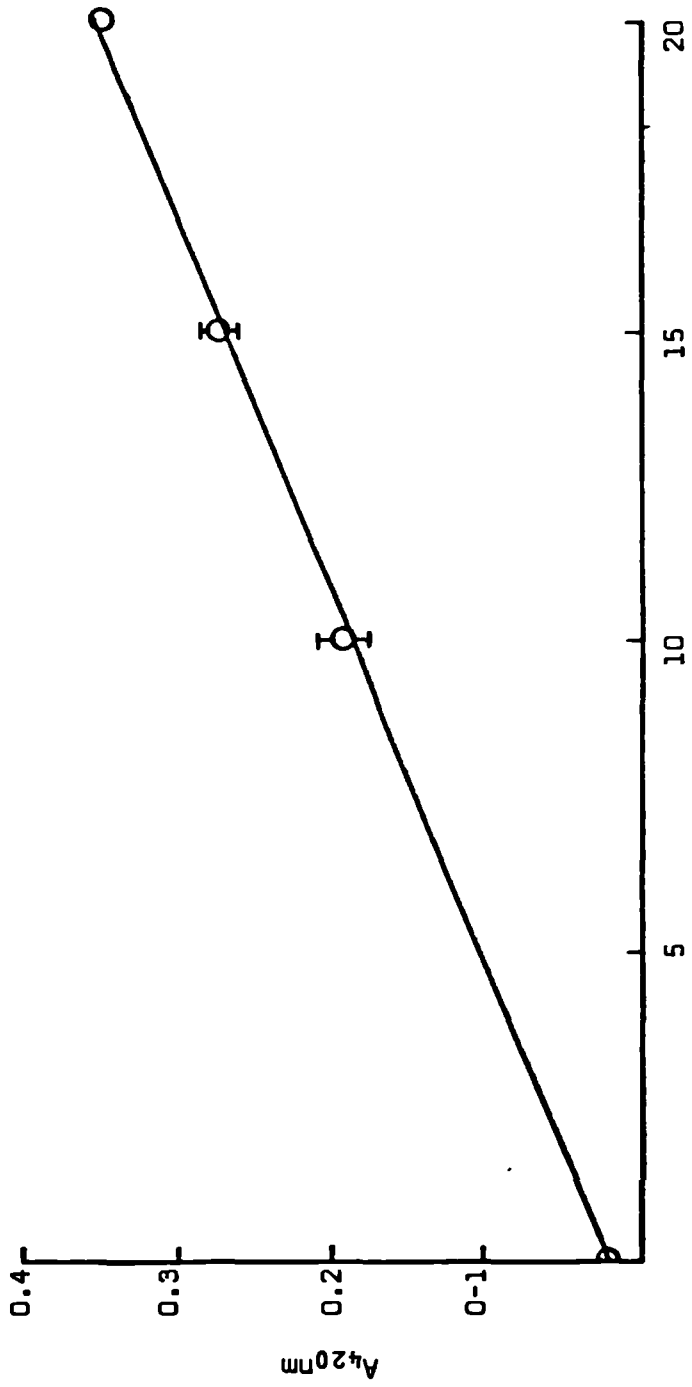
The method was similar to the last experiment except that the substrate was changed to acetonitrile. A linear progress curve was obtained (Fig.7.2) with only a small positive zero-time value. This method was therefore effective in terminating the formation of ammonia from both acetonitrile and acetamide when added to reaction mixtures containing intact cells of the bacterium. The use of Ag ions to terminate enzyme activity was therefore used in subsequent experiments, described in this chapter, to investigate the comparative hydrolysis of different nitriles and amides.

To convert A_{420} nm values, obtained in the following experiments, into µmoles ammonia, standard ammonium sulphate samples (in 0.1M phosphate buffer, pH 7.0) were added to $AgNO_3$ solution and then subjected to microdiffusion in exactly the same way as for experimental samples. The resulting calibration graph (Fig. 7.3) revealed that the A_{420} was directly proportional to ammonia concentration up to an O.D. of at least 1.0. Standard ammonium sulphate samples and reagent blanks were included in every subsequent experiment involving microdiffusion.

7.1.3. Acetonitrilase Activity in Intact Cells of the *Rhodococcus* sp: A Comparison of Both Methods for the Determination of Ammonia.

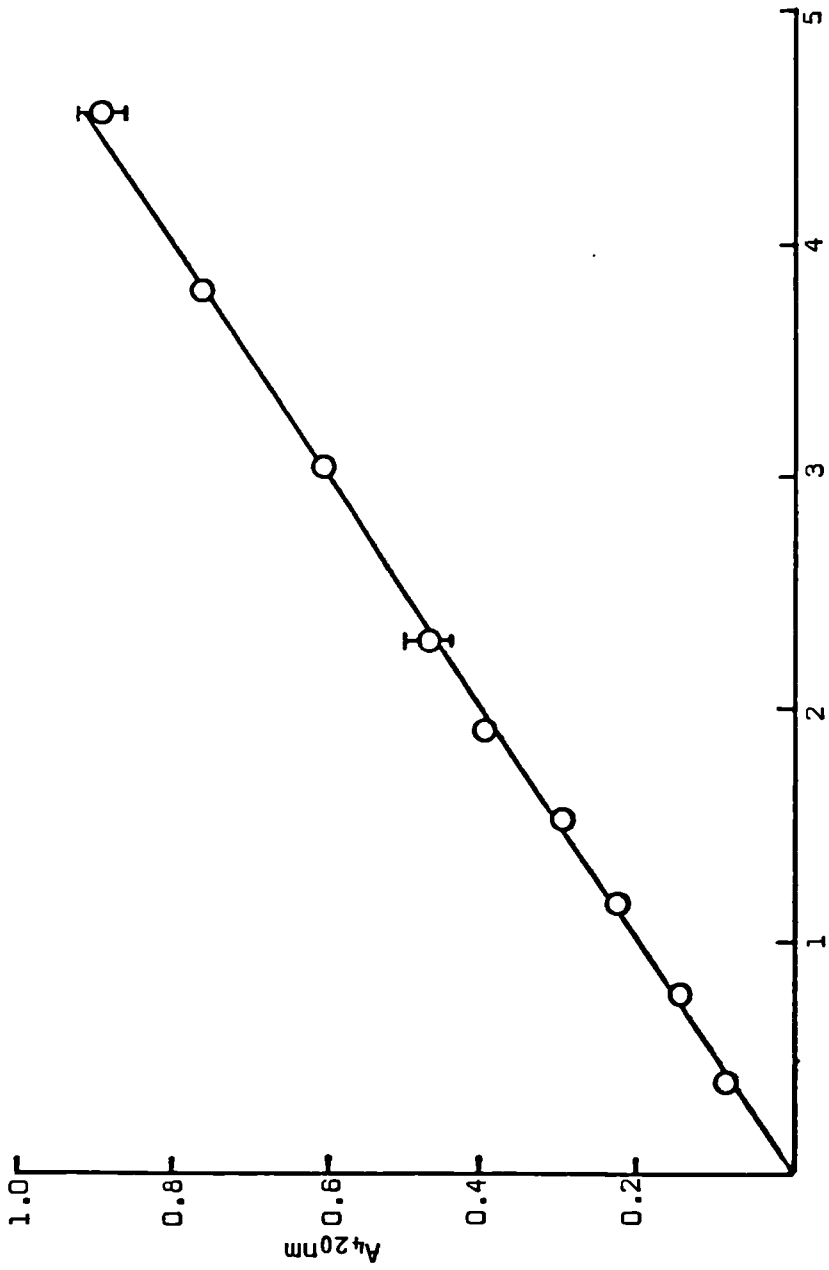
Finally, before adopting the microdiffusion technique, including the use of Ag ions to terminate enzyme activity, ammonia production from the

FIGURE 7.2. Acetontrilase Activity in Cells of the *Rhodococcus* sp. : Termination with Silver Ions.



Experimental details were essentially the same as those described for Fig. 7.1. except the original cell suspension was only diluted 2.5-fold prior to assay and for the nature of the substrate used .

FIGURE 7.3. Calibration Graph for Ammonia After Addition to Silver Nitrate Solution Followed by Microdiffusion and Nesslerisation.



Results are the average A_{420nm} values obtained for duplicate samples less the average A_{420nm} value obtained for duplicate reagent blanks, versus the amount of NH_3 microdiffused (µmoles). Other details are given in the text.

hydrolysis of acetonitrile by cells of the bacterium was determined by both this method and by the direct addition of Nesslers' reagent (Section 2.7.4) without prior microdiffusion. Table 7.3 shows that the nitrilase activity determined by the method involving microdiffusion was 10 ± 8% less than that determined by the direct addition of Nesslers' reagent. Thus the two methods yielded comparable results allowing for the fact that they involved separate reaction mixtures, having different cell concentrations.

7.2. THE HYDROLYSIS OF DIFFERENT NITRILES AND AMIDES BY THE *RHODOCOCCUS* sp. GROWN ON ACETONITRILE.

When considering the concentration of substrate to use for experiments designed to compare the relative rates of hydrolysis of various nitriles and amides, in addition to the results given in Section 5.3.4. two further factors had to be considered. The aromatic amides, benzamide and α-phenylacetamide are only sparingly soluble in aqueous solution and when in saturated solution in the reaction mixture the concentration of benzamide did not exceed 0.05M while that of α-phenylacetamide was approximately 0.04M. Secondly, formamide undergoes spontaneous hydrolysis in the presence of an equal volume of saturated K_2CO_3 solution. The results given in Table 7.4 show that this reaction would result in an excessively high no-enzyme control reading if the formamide exceeded 0.05M.

From a consideration of all these results it was decided to continue using nitriles and amides at a final concentration of 0.05M in reaction mixtures with the exception of α-phenylacetamide which was used at approximately 0.04M.

7.2.1. Hydrolysis of Amides

In the first series of experiments the hydrolysis of ten amides was

TABLE 7.3

Ammonia Production from Acetonitrile Hydrolysis by *Rhodococcus* sp. Cells Determined by Two Different Methods

Method	Nitrilase activity (nmoles NH_3 produced/ ml concentrated cell suspension/minute)
Microdiffusion - followed by nesslerisation	1300 ± 60
Direct nesslerisation	1450 ± 60

The bacterium was grown, harvested and resuspended as for Table 7.2. Reaction mixtures (as detailed in Section 2.7.4 - modification 2) were incubated for 0 and 10 minutes. For the method involving microdiffusion the concentrated cell suspension was diluted 2-fold prior to assay whereas the cell suspension for the method involving direct nesslerisation was diluted 5-fold. The differences between duplicate values obtained for the zero-time and 10 minute "experimental" mixtures were used to calculate enzyme activities.

TABLE 7.4

The Spontaneous Formation of Ammonia from Different Concentrations of Formamide During Microdiffusion

Concentration of formamide solution (M)	A_{420}nm values after nesslerisation
0	0.032 ± 0.008
0.05	0.202 ± 0.017
0.10	0.414 ± 0.012
0.20	0.641 ± 0.079

Samples (1.0ml) of formamide in 0.05M sodium phosphate buffer (pH 7.0) were microdiffused and the ammonia determined as described in Section 2.7.4 - indirect method. Results are the average of duplicate analyses.

investigated using intact cells of the *Rhodococcus* sp. The results (Table 7.5) show that besides acetamide only propionamide and acrylamide were rapidly hydrolysed. Formamide and n-butyramide were slowly hydrolysed as was nicotinamide to a very small extent. The hydrolysis of malonamide, benzamide and α-phenylacetamide could not be detected.

Assuming the amidase is located intracellularly, the negative results obtained could have been due to the inability of a hypothetical permease system to transport malonamide or the aromatic amides. Thus the hydrolysis of these amides was investigated using a suspension of the *Rhodococcus* sp. which had been sonicated for a total of 5 minutes so that between 5 and 10% of the amidase activity was released into the medium. Again there was no detectable hydrolysis of any of these compounds even after 120 minutes incubation with the crude sonicated suspension thus confirming the results obtained using intact cells. The experiment was repeated yet again, this time using a cell-free extract prepared after sonicating a cell suspension for a total of 30 minutes (followed by centrifugation) so that approximately 37% of the amidase activity was released into the medium (see Section 5.2.1). Still there was no detectable formation of ammonia when the extract was incubated separately with the three amides, again over a period of 120 minutes, even though the extract was very active in hydrolysing acetamide.

Therefore it was concluded that malonamide, benzamide and α-phenylacetamide were not substrates of the amidase(s) induced in cells of the *Rhodococcus* sp. grown on acetonitrile.

Progress curve for acrylamide hydrolysis.

It is interesting that the hydrolysis of acrylamide by intact cells and extracts of *P. aeruginosa* (PAC 1) was not linear with time presumably due to inhibition of the enzyme by acrylamide and/or acrylic acid (Kelly

TABLE 7.5

Hydrolysis of Amides by Acetonitrile-grown Cells of the *Rhodococcus* sp.

Amide	Rate of hydrolysis (nmoles NH_3 produced/mg dry wt. cells/minute)
Propionamide	9070 ± 1200
Acrylamide	4540 ± 400
Acetamide	2100 ± 200
Formamide	361 ± 42
n-Butyramide	65 ± 6
Nicotinamide	1.6 ± 0.4
Malonamide	N.D.
Benzamide	N.D.
α-Phenylacetamide	N.D.

N.D., not detected

The bacterium was grown, harvested and resuspended as for Table 7.2 Experimental reaction mixtures (Section 2.7.4 - modification 2) were incubated at 25°C until between 2 - 6% of the substrate had been hydrolysed to ammonia. If this took less that 10 minutes the cell suspension was diluted prior to assay. When no hydrolysis was observed, reaction mixtures were incubated for 30 minutes. Zero-time experimental samples were also analysed in addition to no substrate, no enzyme and boiled controls. All reactions were terminated by the addition of $AgNO_3$ solution (see Section 7.1.2.) Ammonia concentrations were determined after microdiffusion and nesslerisation as in Section 2.7.4. - indirect method. Results were calculated from the difference between the experimental and zero-time sample and are the average of duplicate assays with the exception of the result for acetamide hydrolysis which was the average of 3 independent experiments.

& Clarke, 1962). More specifically, the rate of acrylamide hydrolysis decreased by approximately 64% after 10 minutes incubation compared to the initial rate measured during the first 5 minutes.

Thus ammonia formation from acrylamide hydrolysis was measured at 5 minute intervals using intact cells of the *Rhodococcus* sp. to see if such a non-linear progress curve was also characteristic of this bacterium. To make the experiment comparable to that of Kelly & Clarke (1962), the overall substrate concentration in the reaction mixture was increased to 0.2M. Fig. 7.4 shows that the rate of ammonia formation from acrylamide was constant over the 15 minute incubation period within the limits of experimental error so the *Rhodococcus* enzyme is not progressively inhibited by acrylamide or its hydrolysis product.

Acyl-transferase activity of *Rhodococcus* sp.

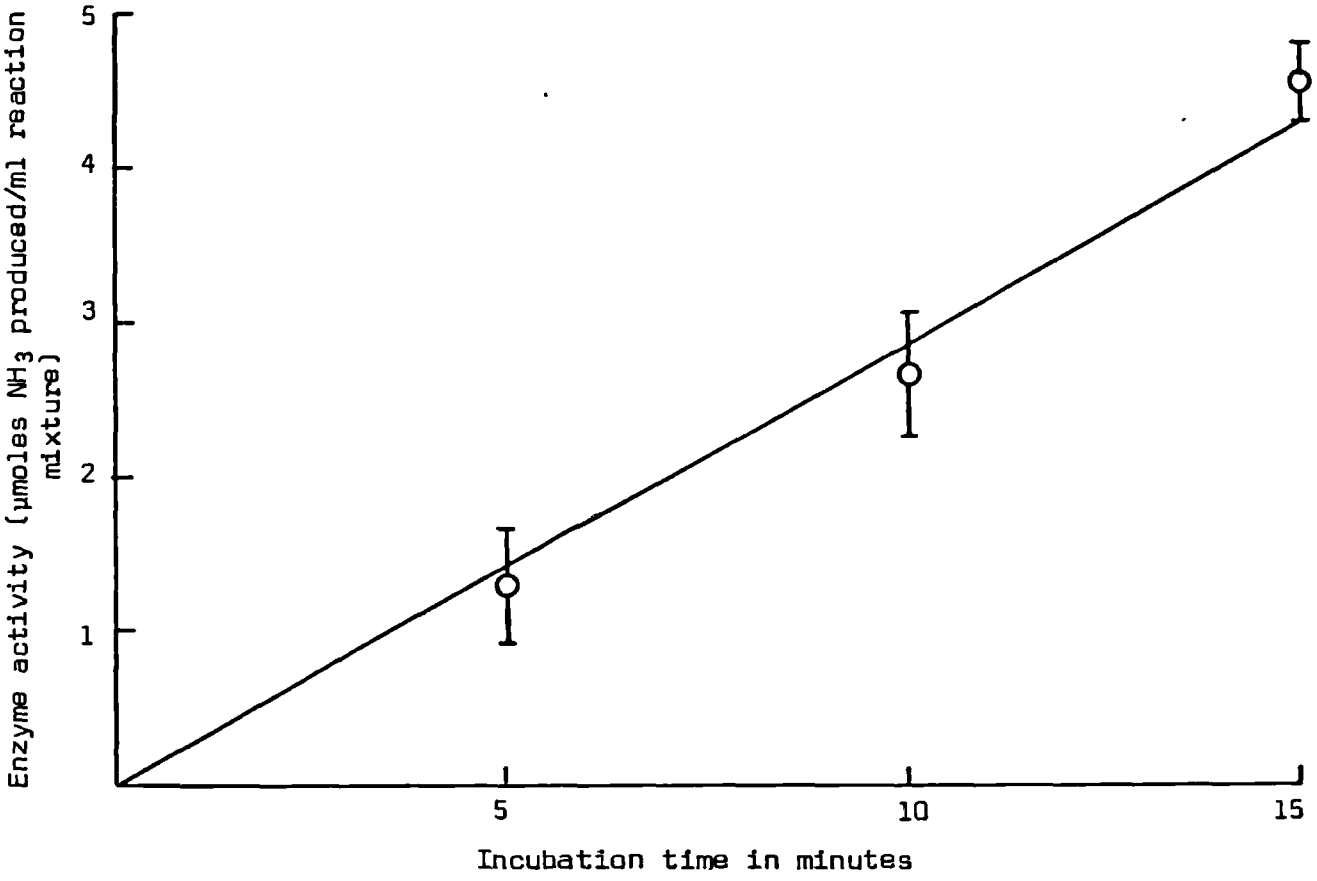
Acetonitrile-grown cells of the *Rhodococcus* sp. were also able to transfer the acyl moiety of acetamide to hydroxylamine thus forming acethydroxamate. The results (Table 7.6) show that the transferase reaction was approximately 9.3 times faster than the hydrolase reaction with acetamide as substrate. If the hydrolase and transferase reactions are catalysed by the same enzyme (i.e. amidase) then whether the rate observed for the transferase activity was the maximum rate possible is not certain since presumably in addition to transferring the acyl moiety of acetamide to hydroxylamine the enzyme would also be involved in hydrolysing the amide.

This is the first time the transfer of the acyl moiety of an amide to hydroxylamine has been demonstrated for a nitrile degrading organism.

7.2.2. Hydrolysis of Nitriles.

In the first series of experiments the hydrolysis of eight nitriles

FIGURE 7.4. The Production of Ammonia by an Intact Cell Suspension of the *Rhodococcus* sp. Incubated with Acrylamide.



Experimental details are the same as those described for Table 7.5 except for varying incubation times.

TABLE 7.6

Comparison of the Hydrolase and Transferase Activities of *Rhodococcus* sp. Cells with Acetamide as substrate

Assay	Activity (nmoles product formed/ mg dry wt. cells/minute)
Hydrolase	2100 ± 200
Transferase	19600 ± 30

The bacterium was grown, harvested and resuspended as for Table 7.2. The product measured for the hydrolase assay was NH_3, and for the transferase assay, acethydroxamate. For details of the hydrolase assay see Table 7.5, and for the transferase assay see Section 2 .7.5.

plus HCN was investigated using intact cells of the *Rhodococcus* sp. The rates at which ammonia was formed from these compounds are given in Table 7.7. A comparison of Tables 7.5 and 7.7 shows that the relative rates of hydrolysis of the nitriles did not parallel those of the corresponding amides. The rate of nitrile hydrolysis, to give ammonia, decreased the greater the number of C-atoms in the molecule. Thus acetonitrile was hydrolysed more rapidly than propionitrile which in turn was hydrolysed more rapidly than butyronitrile. Acrylonitrile, the only other nitrile definitely shown to be a substrate of the nitrilase, was hydrolysed at a rate intermediate between acetonitrile and propionitrile.

Ammonia formation from aminoacetonitrile, N-(2-cyanoethyl)-glycine or 2-amino-1-propene-1,1,3-tricarbonitrile could not be detected after 120 minutes incubation with the concentrated cell suspension. However, from two separate experiments, 3-aminopropionitrile appeared to give slightly positive results; the ammonia production was calculated to be 2.4 $\pm$ 1.4 and 0.4 $\pm$ 0.2 nmoles NH_3 produced/mg dry wt. cells/minute. The average for these two results is 0.93 with a S.D. of 1.29.

An attempt was next made to prove whether or not these negative results were due to permeability barriers : aminoacetonitrile, N-(2-cyanoethyl)-glycine and 3-aminopropionitrile were incubated with a suspension of the *Rhodococcus* sp. which had been sonicated for a total of 5 minutes. Although the crude sonicated suspension readily hydrolysed acetonitrile, no ammonia formation was detected in the other cases even though the incubation period was 120 minutes. In particular, for 3-aminopropionitrile, the ammonia formed above that of the no substrate control was less than the experimental uncertainty.

Despite this evidence it is still possible that the aminonitriles were substrates of the nitrilase: aminoamides might have been formed which

TABLE 7.7

Hydrolysis of Nitriles by Acetonitrile-grown Cells of the *Rhodococcus* sp.

Nitrile	Rate of hydrolysis (nmoles NH_3 produced/mg dry wt. cells/minute)
Acetonitrile	85.9 ± 3.1
Acrylonitrile	27.2 ± 4.2
Propionitrile	2.14 ± 0.06
n-Butyronitrile	0.868 ± 0.003
Hydrogen cyanide	<0.3
3-Aminopropionitrile	N.D.
Aminoacetonitrile	N.D.
N-(2-cyanoethyl)-glycine	N.D.
2-Amino-1-propene-1,1,3-tricarbonitrile	N.D.

N.D., not detected

Experimental details were essentially as described for Table 7.5 except for the majority of substrates (excluding acetonitrile) the incubation period was increased to 1 - 3 hours.

were not susceptible to further hydrolysis by the amidase. The hydrolysis of the aminoamides themselves, by the *Rhodococcus* sp., could not be readily tested since the majority were not commercially available. Thus no conclusions could be made at this stage as to the limited specificity of the nitrilase.

However, it is now known that glycinamide, the amide corresponding to aminoacetonitrile, is spontaneously degraded to ammonia during the microdiffusion step, presumably due to alkaline hydrolysis by the K_2CO_3 (D.O. Gray, personal communication, 1981). Hydrolysis was almost quantitative (>80%) so any glycinamide formed from aminoacetonitrile by nitrilase action should have yielded ammonia whatever the properties of the amidase. This argument, however, is based on the assumption that any glycinamide formed would have been released from the cells either before or after they come into contact with the K_2CO_3. Since this has not been proven, a slight doubt still remains.

Further investigations into the possible hydrolysis of aminoacetonitrile to glycinamide and of 3-aminopropionitrile to 3-aminopropionamide by acetonitrile-grown cells of the *Rhodococcus* sp. have been made by employing the analytical technique of nuclear magnetic resonance (n.m.r.) (D.O. Gray, personal communication, 1981). Even after incubating a concentrated cell suspension separately with these aminonitriles for 5 hours at 25°C no evidence was obtained for the formation of the corresponding amides. It was calculated from the sensitivity of these experiments that aminoacetonitrile and 3-aminopropionitrile could not have been hydrolysed at more than approximately 2% and 4% the rate of acetonitrile, respectively.(Miller & Gray, 1982).

The result given in Table 7.7. for the hydrolysis of HCN to ammonia is an average of 3 independent experiments. In the first two experiments intact cell suspensions were incubated with the substrate solution (KCN in buffer adjusted to pH 8.0) for 120 minutes. The results for these two experiments were 0.28 $\pm$ 0.13 and 0.29 $\pm$ 0.25 nmoles NH_3 produced/mg. dry wt. cells/minute . In the third experiment the cell suspension was sonicated for a total of 5 minutes but not centrifuged. After 120 minutes in the presence of KCN the ammonia released was calculated to be 0.22 $\pm$ 0.15 nmoles NH_3 produced/mg. dry wt."cells"/minute. Thus all 3 experiments gave marginally positive results. The average for the 3 experiments was 0.26 which, like the results for the hydrolysis of 3-aminopropionitrile is really bordering the limits of sensitivity for the assay.

Formamide, the amide corresponding to HCN, is not hydrolysed very

efficiently by the *Rhodococcus* sp. so as a precaution formamide assays were carried out at the same time as the ammonia determinations for both the intact and sonicated cell experiments. The analysis of formamide was carried out as described in Section 2.7.3. In neither case was there any difference between the A_{540} measured for the experimental reaction mixture and the summation of the no substrate and no enzyme controls after cells had been incubated with the cyanide for 120 minutes.

7.3. ENZYME INHIBITION/STIMULATION STUDIES.

7.3.1. Investigations Involving Amides.

The non-substrate amides malonamide, benzamide and α-phenylacetamide and the poor substrate nicotinamide were tested as modifiers of amidase activity using acetonitrile-grown cells of *Rhodococcus* sp. and acetamide as substrate. Three of the potential modifiers were present in reaction mixtures at the same molar concentration as acetamide (0.05M) whereas α-phenylacetamide could only be used at 80% of this concentration because of its poor solubulity. The effects of the potential modifiers were studied in both whole bacteria and in cell-free extracts (prepared after 10 minutes sonication, Section 5.2.1).

From Table 7.8 it can be seen that both nicotinamide and benzamide stimulated acetamidase activity whereas malonamide had no effect at all. α-Phenylacetamide appeared to inhibit ammonia formation by approximately 24% in reaction mixtures containing intact cells whereas no inhibition could be detected when cell-free extracts were used. However, the interpretation of this result is complicated by the fact that α-phenylacetamide came out of solution in the microdiffusion vials and formed a thin film over the surface of the sample. If, in the intact cell experiment, the vials were agitated insufficiently to break the surface film then the diffusion of ammonia

TABLE 7.8

The Effect of the Non-substrate Amides and Nicotinamide on Acetamidase Activity in the *Rhodococcus* sp.

Amide tested as modifier of acetamidase activity	% Inhibition or stimulation	
	Intact cells	Cell-free extract
Malonamide	0	0
Benzamide	+18	+14
α-Phenylacetamide	-24	0
Nicotinamide	+19	+44

The bacterium was grown, harvested and resuspended as for Table 7.2. Acetamidase activity was determined as described in Section 2.7.4 - modification 2 except that an equal concentration of the modifier (overall concentration of 0.05M) was added in addition to, and at the same time as, the acetamide. Experimental reaction mixtures were incubated for 0 and 10 minutes (intact cells) or 30 minutes (cell-free extract) and activities determined after calculating the difference between the two values obtained, in duplicate. In reaction mixtures in which nicotinamide only was incubated with the cells or extract there was no detectable formation of ammonia. Reaction mixtures were terminated and ammonia concentrations determined as for Table 7.5.

onto the acid trap may have been slowed down.

7.3.2. Investigations Involving Nitriles

The two aminonitriles, aminoacetonitrile and 3-aminopropionitrile, were tested as modifiers of acetonitrilase activity using acetonitrile-grown cells of the *Rhodococcus* sp. which had been sonicated for a total of 5 minutes prior to use but not centrifuged. When present at the same molar concentration as acetonitrile (0.05M), aminoacetonitrile caused an 85% reduction and 3-aminopropionitrile a 30% reduction in ammonia formation. The experiment was repeated with exactly the same results. Assuming that the aminonitriles are not hydrolysed by the nitrilase, as indicated by the n.m.r. experiments, then aminoacetonitrile and 3-aminopropionitrile may truely be inhibitors of the enzyme. However, since in this experiment 90-95% of the nitrilase activity was still present in cells, the aminonitriles could have interfered with acetonitrile uptake instead.

7.4. DISCUSSION.

Of the nitriles which supported growth (Table 4.5) only 3-aminopropionitrile was not definitely shown to be hydrolysed by acetonitrile-grown cells of the *Rhodococcus* sp. This suggests either a very slow rate of hydrolysis of the aminonitrile (which could not be detected either by n.m.r. or nesslerisation) or that the bacterium can synthesise more than one nitrilase. In the latter case, the nitrilase responsible for the hydrolysis of 3-aminopropionitrile would not be induced by acetonitrile. Although the *Rhodococcus* sp. could not grow in medium containing acrylonitrile as either the source of C or N, this nitrile was the second fastest substrate to be hydrolysed by acetonitrile-grown cells.

The results presented here appear to be the first study of the comparative rates of hydrolysis of different nitriles by intact cells of micro-

organisms. However, DiGeronimo & Antoine (1976) found that extracts of *N. rhodochrous* LL100-21 hydrolysed acetonitrile about 7 times faster than propionitrile. Cells of the *Rhodococcus* sp. also hydrolysed acetonitrile faster than propionitrile but the ratio of the two rates was much greater, i.e. 40:1.

A more extensive survey of the hydrolysis of various nitriles has been made by Kuwahara *et al.*, (1980) using extracts of butyronitrile-grown cells of *F. solani* (Mn 7030). The following nitriles were hydrolysed to ammonia (in order of comparative hydrolysis rate), n-butyronitrile > malononitrile > glutaronitrile > succinonitrile > adiponitrile > propionitrile > acetonitrile. Acetonitrile breakdown could hardly be detected, so for the mononitriles, the relationship between carbon chain length and hydrolysis rate was the reverse of that seen in the *Rhodococcus* sp.

The same extracts of *F. solani* (Mn 7030) were also shown to hydrolyse the following amides to ammonia (again given in order of comparative rate of hydrolysis), acetamide > propionamide > n-butyramide > formamide ≡ n-valeramide ≡ caproamide. There was only a trace of ammonia with adipamide as substrate whereas malonamide and succinamide were not hydrolysed at all (Kuwahara *et al.*, 1980).

With extracts of *F. solani* (Mn 7030) it is particularly interesting that ammonia was formed faster with n-buryronitrile as substrate than with n-butyramide, the same being true for the adiponitrile/adipamide pair. Moreover, the extract hydrolysed malononitrile and succinonitrile but not the corresponding amides. Therefore, the hydrolysis of these four nitriles by two, sequential, enzymes involving a nitrilase and amidase seems unlikely. It would seem more probable that these nitriles are metabolised directly to the corresponding carboxylic acids and ammonia as is the case for benzonitrile degradation by another strain of *Fusarium solani* (Harper, 1977b) and *N. rhodochrous* (Harper, 1977a).

A strain of *Brevibacterium* R312 has been shown to have a very broad spectrum of nitrilase activity (Arnaud *et al.*, 1976 a,b). Cell suspensions hydrolysed the following types of nitriles, specific examples being shown in brackets: aliphatic mononitriles (e.g. acetonitrile & propionitrile), aliphatic dinitriles (e.g. malononitrile), α and β-unsaturated nitriles (e.g. acrylonitrile), α and β-aminonitriles (e.g. aminoacetonitrile & 3-aminopropionitrile), α-hydroxynitriles, aromatic nitriles (e.g. benzonitrile) and heterocyclic nitriles. The same bacterium could also hydrolyse the amides corresponding to all the nitriles tested with the exception of some α-unsaturated amides (e.g. acrylamide) and the cyclic amides (lactams), (Arnaud *et al.*, 1976c). Similar spectra of nitrilase and amidase activities were shown for other bacterial strains belonging to the genera *Bacillus*, *Bacteridium* and *Micrococcus* (Arnaud *et al.*, 1976 a, b and c). However, since none of these results were given in quantitative terms, a comparison of the relative rates of hydrolysis with those for cells of the of the *Rhodococcus* sp. could not be made.

Not all the amides which could support the growth of the *Rhodococcus* sp. when supplied as the sole source of C and/or N could act as substrates for the amidase present in acetonitrile-grown cells; this was true of malonamide, benzamide and α-phenylacetamide. This suggests that the bacterium is capable of synthesising more than one amidase enzyme.

The amidase induced in acetonitrile-grown cells of the *Rhodococcus* sp. could hydrolyse acrylamide rapidly although this compound could not support growth. Acrylamide may not be an inducer of the amidase but this is an inadequate explanation of its failure to support growth for two reasons. First, the cells used to inoculate the test media were grown on acetate, an amidase inducer, and although resting cells were used some amidase activity may still have been present. Secondly, even if there was not,

acetate was present in the growth medium when acrylamide was tested as a N-source. Thus it appears that either acrylamide, or its hydrolysis product acrylic acid, is toxic to the cells or else acrylamide can act as a repressor of amidase synthesis in this *Rhodococcus* sp. Similar arguments apply to the results obtained for acrylonitrile.

Formamide apparently acted as a N but not a C-source. The obvious explanation is that the *Rhodococcus* sp. could not utilise formate. However, there is an alternative hypothesis: if the starter culture grown on acetate/ $(NH_4)_2SO_4$ lacked amidase and formamide could not induce it, growth would only occur in the presence of the added C-source, i.e. acetate. In this case acetate would be acting primarily as an amidase inducer rather than a C-source. To overcome this ambiguity, the starter culture was grown in succinate/ $(NH_4)_2SO_4$ medium and used to inoculate test media containing formamide with succinate as an additional C-source. Since growth occurred in this medium, formamide must induce the amidase since succinate cannot do so. Thus the obvious explanation of the original observation, that formate cannot act as a C-source, must be the correct one. Similar arguments apply to the utilisation of malonamide and nicotinamide. Benzamide also supported growth of the *Rhodococcus* sp. when present as the sole source of N. Only acetate was tested as the additional C-source here but this was unlikely to induce a benzamidase since acetonitrile-grown cells were unable to hydrolyse benzamide. Therefore, it appears that although benzamide can induce a special amidase responsible for its own hydrolysis, benzoate is inert as a C-source.

Not only the order but also the comparative rates of hydrolysis of the substrate amides to yield ammonia, using intact cells of the *Rhodococcus* sp., bear a striking resemblance to the results obtained by Kelly & Clarke (1962) for induced cells of *P. aeruginosa* (PAC 1), an interesting observation considering the diverse taxonomic positions of the two bacteria. But the resemblance does not stop there; neither malonamide or benzamide were

substrates of the *P. aeruginosa* amidase, a finding in common with that for acetonitrile-grown cells of the *Rhodococcus* sp. It is also interesting to note that glycinamide was inert as a substrate for the *P. aeruginosa* amidase when assayed using whole bacteria or cell-free extracts. In fact, the spectrum of amidase activity for *P. aeruginosa* (PAC 1) was very limited; of the 31 amides tested (including 9 N-substituted amides) only 6 were shown to be hydrolysed (Kelly & Clarke, 1962).

In 1964, Jakoby & Fredericks purified the amidase from acetamide-grown cells of *Pseudomonas fluorescens* and showed that the substrates, in order of declining hydrolysis rate, were propionamide, acetamide and acrylamide. The enzyme could not, however, hydrolyse formamide, n-butyramide, benzamide, alaninamide, ASN, GLN or nicotinamide.

Several species of *Mycobacterium* possess amidase activity which has been attributed to more than one enzyme. For example, Nagayama *et al.*, (1961) looked at a variety of Mycobacteria and found that all the saprophytic strains tested possessed formamidase activity. More detailed investigations using *Mycobacterium* strain 607 revealed that this activity was due to a different enzyme to that catalysing the hydrolysis of benzamide, nicotinamide and L-ASN.

The amidase activity of *M. phlei* was attributed to at least 3 different enzymes; an asparaginase, a nicotinamidase and an amidase which hydrolyses glycinamide (Halpern & Grossowicz, 1957). The relative hydrolysis rates of different amides by extracts of this bacterium was quite different to that observed for cells of the *Rhodococcus* sp., i.e. DL-valinamide > DL-leucinamide > nicotinamide > formamide > glycinamide > L-ASN > acetamide > L-GLN. However, a proper analysis of these results is complicated by the fact that the substrates were used at different concentrations and in some cases the results were indicative of substrate exhaustion or very nearly so.

Mycobacterium smegmatis has also been shown to hydrolyse a variety of amides and again there was some evidence that more than one enzyme was involved, (Draper, 1967). It is interesting that acetamide-grown cells of this bacterium showed a trace of benzamide hydrolysis.

The hydrolysis of aliphatic amides has been shown for many other micro-organisms including a thermophilic *Bacillus* sp. (Thalenfeld & Grossowicz, 1976), *Torulopsis utilis* (Steiner, 1959), *Aspergillus nidulans* (Hynes & Pateman, 1970) and *Chlamydomonas reinhardi* (Gresshoff, 1981). However, of the organisms possessing amidase, so far investigated, the *Rhodococcus* enzyme is most like that of *P. aeruginosa* although several organisms are similar to the *Rhodococcus* sp. in that they appear to contain more than one amidase.

In addition to hydrolysing acetamide, acetonitrile-grown cells of the *Rhodococcus* sp. could also transfer the acyl-moeity of acetamide to hydroxylamine thus forming acethydroxamate and ammonia. The ratio of transferase to hydrolase activity was approximately 9.3:1, while the corresponding ratio for the purified amidase of *P. aeruginosa* was 5:1 (Kelly & Kornberg, 1964). However, cells of *M. smegmatis* gave results contrasting with these in that the hydrolase activity was approximately 18 times the transferase activity (Draper, 1967). Furthermore, with propionamide, n-butyramide or nicotinamide as substrate, the transferase activity was greater for succinate-grown than for acetamide-grown cells; the reverse was true of amide hydrolysis. In *M. smegmatis* the amide transferase and hydrolase are different enzymes: the two activities did not co-purify during $(NH_4)_2SO_4$ fractionation (Draper, 1967). *Mycobacterium avium* was similar in that the nicotinamidase activity was attributed to a different enzyme to that catalysing nicotinotransferase activity (Kimura, 1959 b). However, the transferase and amide hydrolase activities of *P. aeruginosa* did co-purify to homogeneity and so were presumably due to the same enzyme (Kelly & Kornberg, 1964).

Both the non-substrate benzamide and the very poor substrate nicotinamide stimulated the formation of ammonia from acetamide in both acetonitrile-grown cells and extracts of the *Rhodococcus* sp. The stimulation by nicotinamide may be related to its structural similarity to the pyridine coenzyme NAD. It would be advantageous to the organism if oxidised pyridine coenzymes stimulated the amidase and thus the supply of $2\bar{H}$. As mentioned in the results (Section 7.3.1) the effect of the non-substrate α-phenylacetamide on acetamidase activity was difficult to interpret. However, it is still possible that α-phenylacetamide may interfere with the uptake of acetamide by intact cells since this aromatic amide reduced acetamidase activity by 24%.

Malonamide was neither a substrate nor a modifier of the *Rhodococcus* acetamidase whether assayed in whole bacteria or in cell-free extracts. The same was true of the corresponding enzyme in whole cells of *P.aeruginosa* (Kelly & Clarke, 1962). Moreover, malonamide could not be utilised or hydrolysed by *Aspergillus nidulans*, neither did it interfere with the utilisation or hydrolysis of acetamide by this fungus (Hynes & Pateman, 1970).

Aminoacetonitrile and 3-aminopropionitrile inhibited the acetonitrilase activity in a crude sonicated suspension of the *Rhodococcus* sp. by 85% and 30% respectively. Since neither aminonitrile was shown to be a substrate of the nitrilase they were probably acting as either nitrilase inhibitors or by interfering with the uptake of acetonitrile by the intact cells remaining in the sonicated suspension.

CONCLUDING REMARKS

Nitrilase enzymes may be divided into two groups; those which hydrolyse the nitrile to the corresponding amide and those which hydrolyse it directly to the carboxylic acid without liberating the amide. All reports so far indicate that bacteria metabolise acetonitrile by a stepwise hydrolysis involving both a nitrilase which converts acetonitrile to acetamide and an amidase which converts the amide to ammonium acetate. This has recently unequivocally been shown to be the case for the nitrile-degrader *Arthrobacter* sp. J-1 by Asano *et al.*, (1980) who found that the acetonitrilase and acetamidase enzymes did not co-purify. Results presented in this thesis also suggest that the conversion of acetonitrile to ammonium acetate by the *Rhodococcus* sp. involves two enzymes. The bacterium could hydrolyse acetamide and a number of other aliphatic amides suggesting the presence of a distinct amidase enzyme; furthermore the acetonitrilase activity was shown to be far more labile than the acetamidase activity.

The importance to an organism for such an inducible nitrilase as found in the *Rhodococcus* sp. is obscure. There are relatively few known natural nitriles, however as Jallageas *et al.*, (1980) commented nitriles are difficult to isolate and identify so it is possible that a large number exist in nature still awaiting discovery.

Early primitive earth simulations have suggested that both HCN and organic cyanides including α-aminonitriles may have been formed in a "reducing atmosphere" composed of molecules such as methane, ammonia and water (Fox & Dose, 1977). Hydrolysis of such aminonitriles could have produced acids of biological importance. Thus speculations that nitrilase enzymes could have played a part in the early evolution of life have been made (Chamberlain & Mackenzie, 1981).

Aliphatic nitriles do exist in the environment as a result of pollution (section 1.2.1) and nitrilase containing microorganisms may play a role in the natural decomposition of these compounds. Also the nitrilase from *Fusarium*

solani is known to hydrolyse a variety of aromatic nitriles including ioxynil and bromoxynil so this organism may play an important part in the breakdown of certain nitrile herbicides in the environment (Harper, 1977b).

Although the aliphatic acyl-transferase of *Pseudomonas aeruginosa* can catalyse a number of reactions (see section 1.3), the only one of physiological significance is thought to be amide hydrolysis (Clarke & Richmond, 1975). This reaction enables amides to be used for growth as C or N sources or both.

Several amides are known to be common in nature for example the aminoacid amides glutamine and asparagine. Urea is also a common amide which is produced as the end-product of nitrogen metabolism by many animal species and it is of no surprise that microorganisms exist which can utilise it for growth thereby continuing the nitrogen cycle. The heterocyclic amide nicotinamide is a normal cell metabolite (Clarke, 1980) while formamide has been shown to be produced by some pathogenic fungi of cyanogenic plants by hydrolysis of the HCN released by the host (Myers and Fry, 1978). Furthermore, formamide has been reported to be the final product of histidine metabolism in *Aerobacter aerogenes* (Magasanik & Bowser, 1955) and *Clostridium tetanomorphum* (Wachsman & Barker, 1955).

Reports on the presence of amidase enzymes from a variety of organisms is therefore to be expected. Joshi & Handler (1962) purified a nicotinamidase from *Torula cremoris* and suggested that it was involved in a salvage pathway to produce nicotinic acid for the resynthesis of pyridine nucleotide coenzymes. The nicotinamidases from rat and rabbit liver were also thought to be concerned with the synthesis of NAD (Petrack *et al.*, 1965).

Although it is still unclear what significance nitrilase enzymes of the type present in the *Rhodococcus* sp. has to the organism what is becoming clear is the possible utilisation by man of the reactions they catalyse. This potential industrial use of the biological hydrolysis of nitriles was first recognised by Mimura *et al.*, (1969); they isolated a new species of *Corynebacterium* from activated sludge which was capable of growing on aceto-

nitrile as well as several other nitriles. Another *Corynebacterium* species (HR3) was isolated two years later by Fukuda *et al.*, (1971) which formed DL-alanine and DL-valine from the respective α-aminonitriles. Thus by utilising microorganisms a number of economically important products may be obtained from nitriles including α-aminoacids, α-hydroxyacids and highly pure amides, e.g. acrylamide.

The advantages of biological as opposed to the chemical hydrolysis of nitriles include the conversions being carried out at moderate pH and temperature, the avoidance of producing by-products and the possibility of stereoselective synthesis of single enantiomers. Already an acetamidase negative of *Brevibacterium* R312 has been shown to possess a second amidase capable of hydrolysing a number of amides including α-aminoamides, however this enzyme will only hydrolyse L-aminoamides to L-aminoacids (Jallageas *et al.*, 1980).

The observations of Mimura *et al.*, (1969); DiGeronimo & Antoine (1976) and Arnaud *et al.*, (1976a,b,c) that the products of nitrile degradation by whole bacteria are released into the medium may prove to be important for the utilisation of nitrilase catalysed reactions in biotechnology. It enables intact cells to be immobilised on supports rather than the purified enzymes. The results from this thesis suggest that the nitrilase may be difficult to use outside of the cell due to its labile nature although immobilisation may reduce the unstable character of this type of enzyme.

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Yamada, H., Asano, Y. & Tani, Y. (1980). Microbial utilization of glutaronitrile. *Journal of Fermentation Technology* 58 (6), 495-500.

LIST OF PUBLICATIONS.

Publications arising from this thesis are as follows:

1. Miller, J.M. & Conn, E.E. (1979). Cyanide Utilization by Plants. A paper presented at the 1979 Pacific Slope Biochemical Conference, University of Nevada Reno, Nevada. U.S.A.

2. Miller, J.M. & Conn, E.E. (1980). Metabolism of Hydrogen Cyanide by Higher Plants. Plant Physiology 65, 1199-1202.

3. Miller, J.M. & Gray, D.O. (1982). The Utilisation of Nitriles and Amides by a Rhodococcus Species. Journal of General Microbiology 128, 1803-1809.